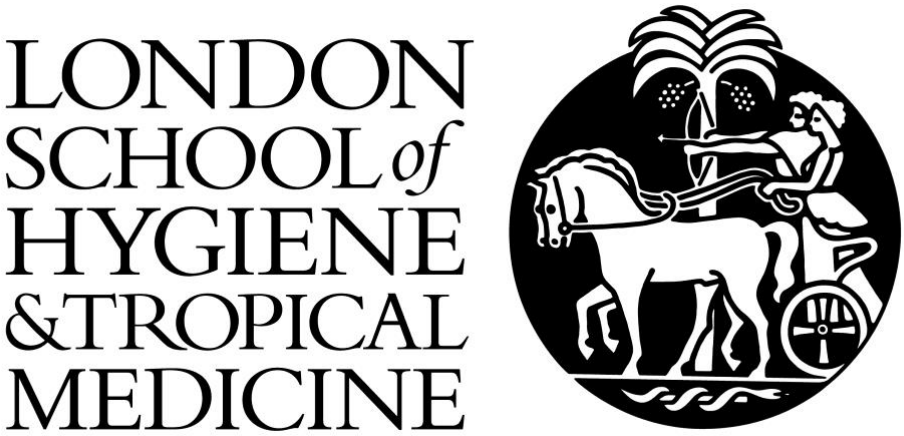

Determinants of Kaposi's sarcoma-associated herpesvirus seropositivity, viral DNA detection and cellular immune responses in Uganda

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A thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy, University of London

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Declaration

I Angela Nalwoga attest that all the research findings presented in this thesis are original work from my PhD. Information obtained from other sources has been referenced. Copyright of this thesis is retained by Angela Nalwoga.

Angela Nalwoga

Abstract

Kaposi's Sarcoma-associated Herpesvirus (KSHV), is a necessary cause of Kaposi's sarcoma (KS), the risk of which increases among people with immune suppression, such as that caused by infection with HIV. KS incidence varies, being highest in places with a high prevalence of KSHV. Controlling KSHV transmission is key in reducing KS incidence. Documented KSHV prevalence is reported to be higher in rural Uganda than has been found elsewhere. This PhD research focused on investigating environmental and immunological factors associated with KSHV antibody responses and viral detection/shedding in blood and in saliva as well as KSHV specific cell-mediated immune responses in Uganda.

ELISA and Luminex were used for antibody measurements, real-time PCR for viral detection and quantification and an ELISPOT assay for cell-mediated IFN-γ response measurement. Infections such as malaria and helminths were the main environmental risk factors analysed.

The factors associated with higher KSHV antibody responses included early age of infection, malaria parasitaemia, low haemoglobin levels and *Schistosoma mansoni* infection. Malaria infection was also associated with higher levels of KSHV DNA in blood while male sex was associated with increased viral shedding in saliva. Children had the highest proportion of individuals with detectable KSHV DNA in blood and in saliva. In relation to IFN-γ production, individuals responded to a wide variety of KSHV peptides without any immune dominance.

In conclusion, KSHV transmission in endemic areas occurs mainly in childhood; this may play a role in the failure to control the virus, leading to increased viral shedding and increased viral transmission. Parasite infections such as malaria and worms may play a significant role in rendering children susceptible to KSHV infection as well as in enhancing reactivation of the virus, increasing lytic replication and, thereby increasing transmission and

pathogenesis.The cell-mediated immune response to KSHV is complex due to the lack of immunodominance.

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Glossary

AIDS	Acquired immune deficiency syndrome
APCDR	African Partnership for Chronic Disease Research
ART	Anti-retroviral treatment
Bcl	B cell lymphoma
BSA	Bovine serum albumin
cART	combination antiretroviral therapy
CCR	Chemokine receptor
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
E	Early
EBV	Epstein Barr virus
EDC	1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide HCl
ELISpot	Enzyme-linked immunosorbent spot
EMaBS	Entebbe Mother and Baby Study
ERK1	Extracellular signal-regulated kinases
ERV	Endogenous retrovirus
FLICE	Fas-associated death domain-like interleukin-1β-converting enzyme
GPC	General Population Cohort
HAART	High active antiretroviral treatment
HCl	Hydrochloric acid
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus

HEB	Hepatitis B virus
HGF	Hepatocyte growth factor
Hhses	Household socio-economic status
HHV	Human herpesvirus
HIF	Hypoxia-inducible factor
HIV	Human deficiency syndrome
HLA	Human leucocyte antigen
HPV	Human papillomavirus
HSV	herpes simplex virus
HTLV	Human T lymphocyte virus
IARC	International agency for research on cancer
ICTV	International committee on taxonomy of viruses
IE	Immediate early
IFN-γ	Interferon gamma
Ig	Immunoglobulin
JNK	c-Jun N-terminal kinases
KICs	KSHV inflammatory cytokine syndrome
KS	Kaposi’s sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late
LAG	Lymphocyte activation gene
LANA	Latently associated nuclear antigen
LaVIISWA	Lake Victoria Island intervention Study on Worms and Allergy-related diseases
LSHTM	London School of Hygiene & Tropical Medicine
MAPK	Mitogen-activated protein kinase
MCD	Multicentric Castleman Disease
MHV	Murine herpesvirus

MIP	Macrophage inflammatory protein
MRC	Medical Research Council
NCAM	Neural cell adhesion molecule
NCI	National Cancer Institute
ORF	Open reading frame
P38	Protein38
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PE	Phycoerythrine
PEL	Primary effusion lymphoma
PI	Principal Investigator
REC	Research and Ethics Committee
RTA	Replication and transcription activator
SIV	Simian Immunodeficiency Virus
Sulfo-NHS	N-hydroxysulfosuccinimide
TLR	Toll-like receptor
UNCST	Uganda National Council for Science and Technology
USA	United States of America
UVRI	Uganda Virus Research Institute
V-cyc	Viral cyclin
V-FLIP	Viral Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein
V-GPCR	Viral G-protein coupled receptor
VOS	Viral Oncology Section
VZV	Varicella zoster virus

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Chapter One: Background

Human Herpesviruses

Organisms from the family herpesviridae are among the oldest animal viruses. They infect a wide variety of vertebrates including mammals, reptiles, birds and fish as well as a few invertebrates. Inclusion in this family was originally dependant on the virion morphology (spherical with four major components: the core, capsid, tegument and envelope as well as linear double-stranded DNA). These viruses are known for the establishment of both lifelong latency and lytic replication by modulating the host immune response [1].

The herpesviridae family is divided into three subfamilies according to the international committee on taxonomy of viruses (ICTV). These include αherpesvirinae, βherpesvirinae and γherpesvirinae [1]. This subfamily classification was originally based on biological criteria such as cell tropism during latency and clinical manifestation of the virus [2]; αherpesvirinae establish latency in neurons, βherpesvirinae in monocyte lineage cells and γherpesvirinae in lymphocytes [1]. These subfamilies have been divided into genera and species based on genomic characteristics including size and structure. Currently, there are nine herpesvirus species that infect humans. HHV-1 (herpes simplex virus -HSV-1), HHV-2 (herpes simplex virus-HSV-2) and HHV-3 (varicella-zoster virus-VZV), the αherpesvirinae; HHV-5 (human cytomegalovirus-HCMV), HHV-6A, HHV-6B and HHV-7, the βherpesvirinae; and HHV-4 (Epstein-Barr virus -EBV) and HHV-8 (Kaposi's sarcoma-associated herpesvirus-KSHV), the γherpesvirinae. EBV belongs to the Lymphocryptovirus genus and KSHV belongs to the Rhadinovirus genus.

Within the family, only γherpesvirinae (EBV and KSHV) are linked to cancer aetiology in humans [3]. EBV was the first infectious agent to be linked directly to cancer (Burkitt's lymphoma). Initial EBV infection is generally asymptomatic, although it can manifest

infectious mononucleosis if infection is delayed into teenage years. Subsequent lifelong infection is usually asymptomatic, but it can be associated with a number of clinical manifestations both in immune competent and immunocompromised individuals. These include non-Hodgkin's lymphomas such as Burkitt's lymphoma and diffuse large B-cell lymphomas, Hodgkin's disease, T cell lymphomas, gastric lymphomas and other lymphoproliferative disorders, as well as nasopharyngeal carcinoma and gastric adenocarcinoma [3, 4]. Identified more recently [5], KSHV has been studied less extensively than EBV. KSHV infection is also generally subclinical, however, can cause neoplasms and lymphoproliferative diseases including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), multicentric Castleman's disease (MCD), and KSHV associated inflammatory cytokine syndrome (KICS) [3, 5-11].

KSHV associated malignancies

All KSHV associated diseases (KS, PEL, MCD and KICS) occur more frequently in HIV infected individuals, although presentation in HIV uninfected people such as organ transplant patients has been documented [13]. Their distribution, therefore, reflects broadly the distribution of both KSHV and HIV infections.

- **Kaposi's sarcoma (KS)**

Of all the malignancies caused by KSHV, KS is the most common. The International Agency for Research on Cancer (IARC) reported 41,799 new KS cases in 2018 from 185 countries [12]. It is among the predominant AIDS-associated cancers and it has a poor prognosis in Africa [13].

Prior to antiretroviral therapy (ART), greater than 30% of AIDS patients developed KS in the USA, primarily men-who-have-sex-with-men (MSM) [11]. ART has drastically reduced the incidence of KS in HIV infected people; in the USA the incidence reduced by 70% with the introduction of ART in the 1990s [13]. However, although ART has greatly reduced KS

incidence worldwide, there are still considerable numbers of KS cases among individuals on ART and KS remains a significant cause of mortality [7, 9, 10]. Chronic antigen stimulation, inflammation and cytokine dysregulation observed in HIV infected individuals on long-term ART may explain the development of KS and other cancers while on ART [13, 14].

The number of individuals living with HIV is increasing worldwide due to the wide use of ART. Despite consequent reductions in KS incidence, as people living with chronic HIV age, this may lead to increases in the incidence of KS in the future, [13] although evidence for this is currently not available. Additionally, in resource-limited settings such as in sub-Saharan Africa (sSA), a substantial number of HIV infected people are diagnosed late or do not receive ART for various reasons. Furthermore, the available KS chemotherapy is toxic and has less favourable efficacy [13]. Thus, mortality from KS remains a major public health problem in sSA [15].

KS has been categorised in the literature into four forms based on the clinical manifestation and epidemiology: classical, endemic/African, iatrogenic/organ transplant-associated and AIDS-associated/epidemic KS. Classic KS occurs in HIV uninfected people, mainly in older men from the Mediterranean region and tumours tend to be localised to the lower extremities. Endemic KS occurs in HIV uninfected Africans (primarily men, although some few cases have been reported among women). Iatrogenic KS occurs in individuals on immunosuppressive drugs due to organ transplantation while AIDS-KS occurs in those with HIV infection. AIDS-KS is the most common and most aggressive of all KS forms. KSHV has been isolated from all four clinical subtypes of KS and all four forms have similar histopathologic presentation [13].

The clinical course of KS is varied. Spontaneous improvement or worsening has been observed in some patients after correcting the underlying immunosuppression. For instance, immune reconstitution inflammatory syndrome associated with tumour development or

worsening after ART initiation has been documented [13] and, transplant patients can undergo spontaneous regression following changes to immune suppression regimen. Likewise, some patients present with indolent patterns while others have aggressive forms [16]. Generally, KS manifests as multifocal skin lesions, nodules or macules. These lesions are mainly on the lower extremities; possibly due, at least in part to relative hypoxia caused by lower skin perfusion pressure [17]. Oedema, ulceration, bleeding, pain and secondary infections may be associated with KS lesions. In classic KS these lesions usually are limited to the skin and can be indolent; but in more aggressive KS forms there is involvement of a wide range of organs and tissues including the oral mucosa and gastrointestinal tract, lungs, bones, and lymph nodes. Pulmonary KS is the leading cause of mortality due to KS [16].

The pathogenetic mechanisms leading to KS are not fully understood. Potential mechanisms include KSHV interfering with both the innate and adaptive immunity to induce proliferation, halt apoptosis, cause angiogenesis, and upregulate certain cytokine production leading to oncogenesis in infected endothelial cells. KSHV- transformed endothelial cells have an altered morphology and are termed spindle cells. Although the proportion of spindle cells expressing lytic genes is only 1-3% in KS, both latent and lytic KSHV proteins seem to play a role in KS and other KSHV malignancies. For example, lytic ORF74 encodes a viral G protein-coupled receptor (vGPCR) that is vital in causing angiogenesis in KS [13]. Most KSHV infected cells in KS lesions exhibit latent infection and latency-associated nuclear antigen (LANA) from ORF73 is expressed in all KSHV infected spindle cells. Therefore, KSHV LANA-1 staining of spindle cells of biopsies is used for KS diagnosis [13, 16, 17].

- **Primary effusion lymphoma (PEL)**

Like KS, PEL mostly occurs in HIV infected people but much less frequently than KS. It is rare, although in Africa it is probably underreported due to diagnostic challenges, including difficulties in obtaining specialised hemopathology support. It is an aggressive mature monoclonal B cell lymphoma with very poor prognostic outcomes. PEL occurs as body cavity

effusions but non-cavity effusions have also been observed on rare occasions. It produces pleural, peritoneal, and pericardial lymphomatous effusions with pleural effusions being predominant. Unlike KS and MCD, PEL tumour cells may contain both KSHV and EBV viruses. All PEL cells contain KSHV while only 80% of them also contain EBV. PEL manifests as an inflammatory disorder with elevated serum inflammatory cytokines, ferritin and IgE antibodies. Long-term remission requires both ART and chemotherapy regimens. However, this combination causes long-term remission in only about 40% of patients [13, 16, 18].

- **KSHV-associated multicentric Castleman's disease (MCD)**

KSHV MCD is a rare B cell lymphoproliferative disease occurring in HIV infected people. Unlike other KSHV malignancies, MCD incidence has not decreased with the increased use of ART. It is also likely underreported in Africa due to underdiagnosis. It is characterised by inflammatory symptoms including fever, night sweats, cachexia and weight loss. In addition, patients have lymphadenopathy, splenomegaly and oedema as well as respiratory, gastrointestinal, dermatological and neurological symptoms. LANA stained plasmablasts of the lymph nodes normally confirm the diagnosis of MCD. It is usually fatal within two years following diagnosis if not treated. Furthermore, long-term remission with therapy has not been documented. KSHV viral load tends to increase with increased disease manifestation and decrease with remission [13, 16, 18].

- **KSHV inflammatory cytokine syndrome (KICS)**

This is the newest observed syndrome associated with KSHV. It occurs in HIV infected people, normally in those with KSHV malignancies (KS or PEL). It has not been studied extensively, and currently, its treatment is limited to specialised centres investigating experimental regimens. It is characterised by an MCD-like severe inflammatory syndrome without lymph node involvement typical of KSHV MCD. Usually, patients with underlying KS or PEL are at a very high risk of mortality if they develop KICS [13, 16, 18]. Understanding

the biology of KSHV is central to developing interventions for preventing and treating this and other KSHV-associated diseases.

KSHV genome and life cycle

The γherpesviruses virion, of which KSHV is a member, have linear double-stranded DNA genomes of about 172Kb [19]. The KSHV genome is made of a long unique coding region of about 140Kb flanked by terminal repeat sequences [20], which features over 90 documented open reading frame (ORF)s [21]. The nomenclature of the KSHV ORFs was based on the prototype herpesvirus saimiri (HVS), a non-human primate Rhadinovirus closely related to KSHV and the map orientation of the KSHV genome is similar to that of herpesvirus saimiri (HVS) [22]. The KSHV genome possesses both conserved ORFs within the γherpesviruses subfamily and unique KSHV genes. The conserved ORFs were originally named ORF1 to ORF75. Of these, 66 are homologs of HVS ORFs [22]. The unique ORFs were originally named K1 to 15. More recently additional unique ORFs have been identified and added including K4.1, K4.2, K8.1, K10.1, K10.5, K10.7, K11.1, and K14.1. Also, K13 and K8 were observed to have homologs in other γherpesviruses [21]. The homolog families of genes encode mostly viral structural and metabolic proteins as well as DNA replication proteins [22]. Unique KSHV genes as well as conserved ORFs encode homologs to human cellular proteins [23]. These homologs are responsible for inhibition of apoptosis, cell cycle shutdown and evasion of host immunity to support tumorigenesis, pathogenesis and survival of the virus [20, 22-24]. KSHV ORFs encoding these cellular homologs include K2 (viral IL6), K9 and K10.5 (viral interferon regulatory factors), ORF13 (virus-encoded family of FLICE inhibitory protein- vFLIP), ORF16 (Bcl-2 homolog), ORF4 (proteins similar to complement binding proteins CD21), ORF14 (NCAM-like adhesion protein), ORF72 (cyclin D homolog), ORF74 (IL8 like G protein-coupled receptor- vGPCR), K6 (viral MIP-1), K4 (viral MIP-2), K4.1 (viral MIP-3) and ORF72 (D type cyclin homolog) [20, 22, 23].

Like other herpesviruses, KSHV undergoes two stages of its life cycle: the lytic and the latent stage [25]. KSHV genes are expressed sequentially within the two transcriptional programs (the latent and lytic programs). During primary infection, there is active replication of the virus, leading to infection of B cells and epithelial cells, as well as, potentially, a number of cells types including, monocytes, macrophages, dendritic cells and endothelial cells. Various receptors, such as DC-SIGN, integrin, certain receptor tyrosine kinase of the Eph family and heparin sulfate are used for viral attachment and entry into different host cells [26]. This is followed by lifelong latency, when the virus resides in specific immune cells, expressing only a few proteins. Among infected cells, a small proportion of $CD19^+$ peripheral B cells have been shown to serve as a long-term reservoir of KSHV [27]. The latent infection is tightly regulated by the host immune response and viral proteins [28].

- **Latent Infection**

During latency, the virus exists in infected cells as multiple copies of an extrachromosomal circular episome with restricted latent gene expression and without production of progeny [28, 29]. The lytic promoter sequences are silent during latency [21]. The active latent genome region encodes for the following: ORF73/LANA-1, ORF72/viral cyclin (v-cyc), ORF71/v-FLIP and ORFK12/Kaposin and 12 miRNAs. Low levels of K1, vIL6 and K15 transcripts are also present, while vIRF3 is detected in infected B cells, [24, 29-32]. During this phase, KSHV relies on the latently associated nuclear protein (LANA)-1 encoded by ORF73 for maintenance of latency as well as genome persistence. Therefore, ORF73 is the most frequently expressed latent protein. It is expressed in tumour cells of KSHV associated malignancies, making it a suitable diagnostic marker [23]. ORF73/LANA is responsible for both latent viral episome DNA replication and segregation to daughter cells during mitosis. To achieve this, LANA tethers the KSHV episome onto the host chromatin via histone H1 during mitosis [29, 33]. LANA also hinders lytic replication by negatively regulating transcription of lytic genes to establish latency [31]. Other latent transcripts play significant roles as well; these include v-cyc, which promotes cellular proliferation, vFLIP which

enhances cell proliferation and inhibits apoptosis, Kaposin B, which increases cytokine expression promoting tumour microenvironment, vIL-6 which inhibits apoptosis to allow cell survival, vIRFs which interfere with the host interferon signalling pathway leading to immune escape of KSHV, and viral miRNAs, which regulate viral lytic genes and cellular survival pathways, [23, 24, 29, 31, 32, 34]. The latency program also plays a significant role in tumorigenesis; for instance, over 95% of spindle cells in KS are latently infected, with only 1-3% of the cells going through lytic replication [31, 35, 36]. Nevertheless, the role of lytic replication in tumorigenesis and virus propagation is essential [28].

- **Lytic activation**

During the lytic cycle, the virus enters into another well-ordered program of viral gene expression, following a sequence of events including lytic reactivation, lytic DNA replication and virion production. Upon reactivation from latency, a full repertoire of lytic viral genes are activated in a temporally regulated manner, leading to transcriptional activation of three classes of lytic genes: Immediate early (IE), early (E), and late (L) genes [37-40]. The Immediate early genes govern the transition of the KSHV genome from latency to the lytic phase and are ORF50/RTA, ORF45, K8α, K8.2, K4.2, K4.1, K4, ORF48, ORF29b, K5 and ORF70. The major single IE protein, RTA, acts as the essential latent/lytic switch that redirects KSHV to enter the lytic replication program required for viral spread and KS tumorigenesis [40]. Additionally, IE genes encode for viral proteins directly involved in gene transcription, cellular modification and viral replication [28]. Early genes including K8, K5, K2, K12, ORF6, ORF57, ORF74, K9, ORF59, K3, ORF37, K1, K8.1A, ORF21, vIL6, PAN RNA, vIRF-1, K1, and K15 are required for DNA replication and gene expression [28], as well as other individual functions, mentioned previously. The late lytic genes encode for mainly structural proteins (glycoprotein-B and K8.1) and capsid protein required for assembly and maturation of the virion [28].

KSHV genotypes

Using variable genes unique to KSHV such as K1 and K15, KSHV has been further classified into genotypes. Several research groups [41-44] have identified KSHV genotypes and their distribution using samples from described patients with KSHV associated malignancies. Using nucleotide sequencing of the K1 variable regions one and two, five major genotypes and 15 clades have been identified [42]. The K1 gene has up to 30% amino acid variability. Generally, the distribution of these genotypes is as follows: genotype A is predominant in western Europe and North America, B in Africa, C in the Middle East and Mediterranean Europe, D in Pacific Islands, South Asia, Australia, Taiwan and E in South America among indigenous people [41-44]. Variants in the K15 gene are classified as P, M and N types [42]. The different genotypes have not been correlated with a specific disease or severity. KSHV co-evolved with the human species, as did other herpesviruses that are known to co-evolve with their host species. It seems likely to have migrated with the human population to different parts of the world from Africa [43, 44]. There has been relatively less research on KSHV genotypes in Africa, where the diseases and infection are endemic.

KSHV distribution

Unlike the other human herpesviruses, which are ubiquitous, KSHV has a distinct geographical distribution, which varies within and between regions. The prevalence of KSHV is highest in sub-Saharan Africa (generally >30%), intermediate in the Mediterranean region (>10%), and low in the USA, northern Europe and Asia (<10%) [45-48]. In the USA and northern Europe, KSHV prevalence is elevated in men who have sex with men (MSM) and recent immigrants from Africa, compared to the general population. A high prevalence has also been reported among indigenous Amerindians in South America [49-51], although the reported incidence of KS is low. In sub-Saharan Africa, a prevalence ranging from 20% to 80% has been reported [15, 52, 53]. In Uganda specifically, other studies have reported a KSHV prevalence of 50% to 60% [54-58]. Data from our own studies have reported a KSHV prevalence of 69% among women from an urban area, and an increasing prevalence with

age among children, reaching about 15% by age five years [59-61]. Newer data from a rural cohort in Uganda shows that participants have amongst the highest prevalence of KSHV ever reported (~90%), with the infection occurring mainly in childhood [62]. The reason for the distinct global geographical distribution of KSHV is currently unknown. It is, therefore, essential to understand risk factors for high KSHV transmission in high prevalence settings such as rural Uganda; modifiable risk factors may offer opportunities for reduction of transmission and consequent disease. This PhD research aimed at addressing this question.

KSHV transmission

The primary route of transmission of KSHV is via saliva, especially in KSHV endemic regions [27, 63]. In sub-Saharan Africa, infection with KSHV can occur early in life [64], with the peak age of acquisition in some studies being between 6-12 years of age [65, 66]. In low prevalence areas, adult acquisition is more common, particularly sexual transmission among MSM, which might also be due to salivary exchange as well; KSHV can also be acquired via blood transfusion [27] however it is unlikely that these transmission modalities have significant relevance for the very high transmission rates of KSHV observed in sub-Saharan Africa [15, 27]. It is important to note that factors affecting KSHV transmission might differ from the ones affecting KSHV pathogenesis. In this PhD thesis, viral shedding in saliva has been used to investigate KSHV transmission while viral detection in blood has been used as a measure of viral reactivation and subsequent risk of disease.

KSHV risk factors

KSHV is necessary but not sufficient to cause KS and other associated malignancies. Both viral latency and lytic activation appear to play a role in the development of KS [67]. Lytic replication is important for viral propagation as well as transmission and critical for KSHV induced tumorigenesis [28]. Biomarkers of KSHV reactivation are increasing KSHV antibody titres [68, 69], and increasing KSHV viral load in blood. There are a number of factors that have been associated with KS development and/or reactivation of KSHV from latency,

including HIV infection and other forms of immunosuppression, as well as oxidative stress, inflammatory cytokines, hypoxia, viral co-infections, parasite infections, exposure to traditional phytotherapeutics ("oncoweeds"), volcanic soils and other soil types [28].

- **Immunosuppression and viral infections**

KSHV reactivation and aggressive forms of KS are seen in individuals with immunosuppression. HIV infection acts indirectly through suppression of KSHV-specific T cell responses [70] and induction of inflammatory cytokines [71]. Antiretroviral therapy is associated with regression of KS and decreasing KSHV detection, and this is accompanied by the restoration of numbers and functions of KSHV-specific $CD4^+$ and $CD8^+$ T cells [40, 72, 73]. KS development in KSHV infected individuals after solid organ transplant is linked to calcineurin inhibitors. This class of drugs prevents organ rejection by immunosuppression largely through T cell inhibition. Loss of the KSHV-specific T cell repertoire has been demonstrated with calcineurin inhibitors, with recovery and remission of KS after treatment is discontinued [74]. The majority of post-transplant KS patients are KSHV seropositive prior to transplantation, suggesting that reactivation of the latent virus is responsible for the disease [75, 76].

Other viral infections that have been associated with KSHV lytic replication include HSV-1 [77], HSV-2 [78], HCMV [79], HHV6 [80], HHV7 [81] and Human papillomavirus (HPV) [82]. These viruses are relatively ubiquitous in all human populations and, they may play a role in reactivation of KSHV where it exists.

- **Hypoxia, oxidative stress and reactive oxygen species**

Low tissue oxygen concentration or hypoxia induces accumulation of hypoxia-inducible factor (HIF-1α/2α) and has been linked to the initiation and progression of KSHV associated cancers. Hypoxia has been shown to stimulate reactivation of KSHV *in vitro* [83-86] and, has also been shown to affect the pathogenesis of other oncogenic viral infections including

Epstein-Barr virus (EBV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human papillomavirus (HPV) and Human T cell lymphotropic virus (HTLV)-1 [28].

Reactive oxygen species such as hydrogen peroxide have been shown to mediate KSHV reactivation from latency [28]. Hydrogen peroxide induces KSHV lytic replication in KS tumours by activating the ERK1/2, JNK and P38 mitogen-activated protein kinase (P38 MAPK) pathways [87]. Hypoxia and inflammatory cytokines mediate spontaneous KSHV reactivation and lytic replication [28].

- **“Oncoweeds” and volcanic soils**

KSHV prevalence varies geographically. In most parts of Africa, the incidence of KS mirrors the prevalence of KSHV. In The Gambia, a high prevalence of KSHV has been reported but a relatively low incidence of KS [46]. Partly based on these observations, environmental factors such as ‘oncoweeds’ have been hypothesised to reactivate KSHV. Supporting this hypothesis, a number of plant species have been shown to reactivate KSHV *in vitro*. The majority of these plants are from KSHV endemic areas and may be utilized in herbal medicine or other traditional practices [88]. KS incidences have been associated with volcanic soils. The fine volcanic soils have been shown to damage lower limb lymphatics causing Podoconiosis, the damage on lower limb lymphatics may explain the association between KS incidence and volcanic soils [89, 90] although data on this are not conclusive.

- **Parasites**

Parasite infections including malaria and helminth infections have been hypothesized as potential co-factors for KSHV transmission and infection. Malaria is one of the leading cause of death due to a single infectious agent worldwide. In 2018 WHO reported 219 million malaria cases in 87 countries with 435,000 deaths. The majority (92% of cases and 93% of death) of the malaria burden is in Africa. Efforts to eliminate malaria in sub-Saharan Africa have not been successful, however, reduction in incidence has been documented. Malaria is

caused by protozoan parasites of the genus Plasmodium which is transmitted by the female Anopheles mosquito vector. There are five Plasmodium species that cause malaria in humans, including *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P knowlesi*. Of these, *P. falciparum* is the most common in Africa (responsible for 99.7% of malaria cases) and it is associated with severe forms of malaria, causing severe anaemia and cerebral malaria in children below the age of five.

Natural immunity to malaria involves mainly antibody responses to the blood stage parasites [91, 92]. Cellular and innate responses have also been observed [92, 93]. Protective immune response to malaria is multifaceted, partly due to the complex life cycle of the parasites, involving numerous immunogens which are genetically diverse [94]. Additionally, immune dysfunctions, particularly immunosuppression caused by malaria infection, have been observed. Immunosuppression associated with malaria infection has been attributed to dendritic cell (DC) dysfunction, upregulation of regulatory T cells [95-97], and T and B cell exhaustion [98]. During acute malaria infection, circulating mature DCs significantly reduce in number, leading to the accumulation of immature DCs as well as impairment of DC function [95, 97]. The ratio of plasmacytoid to myeloid DCs is also affected by a great reduction in plasmacytoid DCs [95]. The central role of DCs in antigen presentation can lead to impairment of adaptive immune response if they are dysfunctional. Plasmacytoid DCs play a major role in innate antiviral immunity by producing type 1 interferons. Therefore, if reduced in malaria infection, it could have effects on viral-specific immunity. Expansion of regulatory T cells and increased levels of IL-10 have also been documented in malaria infection [96]. This immune regulation affects T helper responses causing some form of immunosuppression [95, 96]. Additionally, repeated malaria infections have been shown to suppress T cell immunity [98]. This immunosuppression caused by malaria infection has been shown to affect other herpesvirus coinfections including VZV, HSV-1 and EBV by causing reactivation of these viruses [99-101]. Furthermore, It is notable that malaria is

known to affect immune control of EBV, increasing the risk of Burkitt's lymphoma among children living in malaria endemic regions [102, 103].

The survival of parasites is dependent upon their ability to interfere with host immune function [104], in turn compromising the host-virus equilibrium in KSHV infection [105]. Epidemiological studies from Italy report substantial declines in KSHV seropositivity and KS incidence in association with the eradication of mosquitoes and the associated decline of malaria [106]. Adaptive immune response dysfunction associated with malaria infection may cause KSHV reactivation, increasing viral replication and shedding in saliva, while innate immune dysfunctions in malaria may render KSHV uninfected children susceptible to infection during acute malaria episodes. We have previously reported an association between malaria exposure, malaria parasitaemia and KSHV seropositivity in women and children in Uganda [60, 61, 107]. Further investigations of malaria KSHV coinfections are reported in this thesis.

We also hypothesise that helminth infections may affect co-infections such as KSHV, due to immunomodulation. Soil-transmitted helminths such as hookworm, as well as Schistosoma, are common in low-income countries, often affecting the poorest people [108]. These parasites have co-evolved with *Homo sapiens* and have therefore devised means of coexistence with the host's immune system, by provoking immunosuppression [109]. A (Th) 2 oriented immune response is necessary for helminths clearance. It has been observed that developing a (Th) 2 response as opposed to a (Th)1 response, important in virus control, interferes with anti-viral immunity. Furthermore, immunoregulation associated with helminth infestations also reduces the (Th)1 response important in controlling viral infections [110, 111]. This association has been demonstrated in mice, where Schistosoma lead to the reactivation of γherpesviruses in *vivo* and *in vitro* [111].

Immune response to KSHV

KSHV evades host immune responses so as to allow its long-term survival. Latency is one of the mechanisms of avoiding viral clearance by immune cells and is achieved by suppression of expression of most viral proteins, essentially hiding the virus from recognition. Other mechanisms involve the expression of various viral genes which are host cellular homologs and interfere with host immune responses. These proteins cause suppression of MHC-1 and MHC-11 molecules, impairment of dendritic cells function, down-regulation of co-stimulatory molecules, and induction of inhibitory cytokines [105, 112] all of which favour viral survival and oncogenesis.

The protective immune response to KSHV is not well documented. Yet it is vital to understand these responses to allow the development of interventions for controlling viral transmission and disease risk. An effective immune response is required to maintain latency, control viral shedding (thereby preventing transmission), and to prevent disease pathogenesis. HIV infection greatly increases the risk of developing KS and other KSHV associated malignancies. This is due to the loss of $CD4^+$ T cells in infected individuals, suggesting a pivotal role for T cell immunity in the control of KSHV infection. In murine models, Murine herpesvirus (MHV)-68, a γherpesvirus similar to KSHV, has been shown to be controlled by both $CD4^+$ and $CD8^+$ T cell responses [113]. Both $CD4^+$ and $CD8^+$ T cells promote the clearance of productive replication during acute MHV68 infection [114, 115]. CD8 T cell responses (in mice) persist for life and prevent reactivation of the virus through the production of perforins and IFN-γ.

In humans, CD8 T cell responses have been better studied compared to CD4+ cell responses [116]. Although results have been contradictory, the majority of studies suggest protective effects of cellular immune responses against KSHV for KSHV-associated malignancies [116]. KS tumour regression was correlated with improvement in KSHV cellular immune responses [73, 117]. Because KSHV encodes over 86 proteins, the main challenge

in investigating cellular responses is the selection of the antigens to use. However, the identification of immunodominant epitopes for both CD4+ and CD8+ KSHV responses has not been possible.

The protective effect of humoral responses to KSHV still requires investigation. However, high levels of antibody to KSHV have been shown to predict KS development [118], and high antibody titres to KSHV are associated with viral reactivation and viral shedding in saliva [68, 69]. In addition, neutralizing antibodies, but not total antibodies were observed to be lower in KS patients compared to controls [119]. This suggests that infection with KSHV may lead to the production of high levels of antibodies which play little role in protection from disease pathogenesis. This PhD research is aimed at investigating determinants for KSHV seropositivity, viral DNA detection in blood and in saliva as well as cellular immune responses.

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Chapter Two: General Methods

Study designs, settings and populations

The work presented in this thesis was nested within three long-standing prospective studies at the Medical Research Council (MRC)/Uganda Virus Research Institute (UVRI) and London School of Hygiene & Tropical Medicine (LSHTM) Uganda Research Unit. These are the General Population Cohort (GPC), the Lake Victoria Island Intervention Study on Worms and Allergy-related diseases (LaVIISWA) and the Entebbe Mother and Baby Study (EMaBS). These three studies provided three different populations with varying prevalence of possible risk factors. The GPC is a very rural population, the Lake Victoria island communities where LaVIISWA was carried out, are unique due to their high HIV prevalence, high *S. mansoni* infection and very poor health services, while EMaBS, from Entebbe, is a relatively urban population with a few people from peri-urban neighbourhoods. Below are the brief descriptions of each in turn.

- **The General Population Cohort (GPC)**

GPC was established in 1989 by the Medical Research Council in collaboration with the Uganda Virus Research Institute with funds from the UK Medical Research Council and UK Department for International Development [1]. The GPC is located in southwestern Uganda, in Kyamulibwa sub-county of Kalungu district, 40km from the shores of Lake Victoria [2, 3] and 16km from the trans-African highway (Figure 1) [4, 5]. The GPC has a population of 22,000 people scattered across the countryside in 25 adjacent villages [5]. Administrative boundaries, as opposed to socio-economic centres, define the village sizes [6]; the smallest village has 300 people while the largest has 1500. It is a rural community with semi-permanent structures built from locally available materials. The area has no tarmac road, making accessibility during the rainy season difficult [7]. The community is relatively stable, homogeneous and young, with 90% of the people less than 50 years of age. Only about 13% of the residents attain education beyond primary level. The main occupation is

subsistence farming along with fish trading. Matooke (plantain bananas) and groundnuts are the staple food [7].

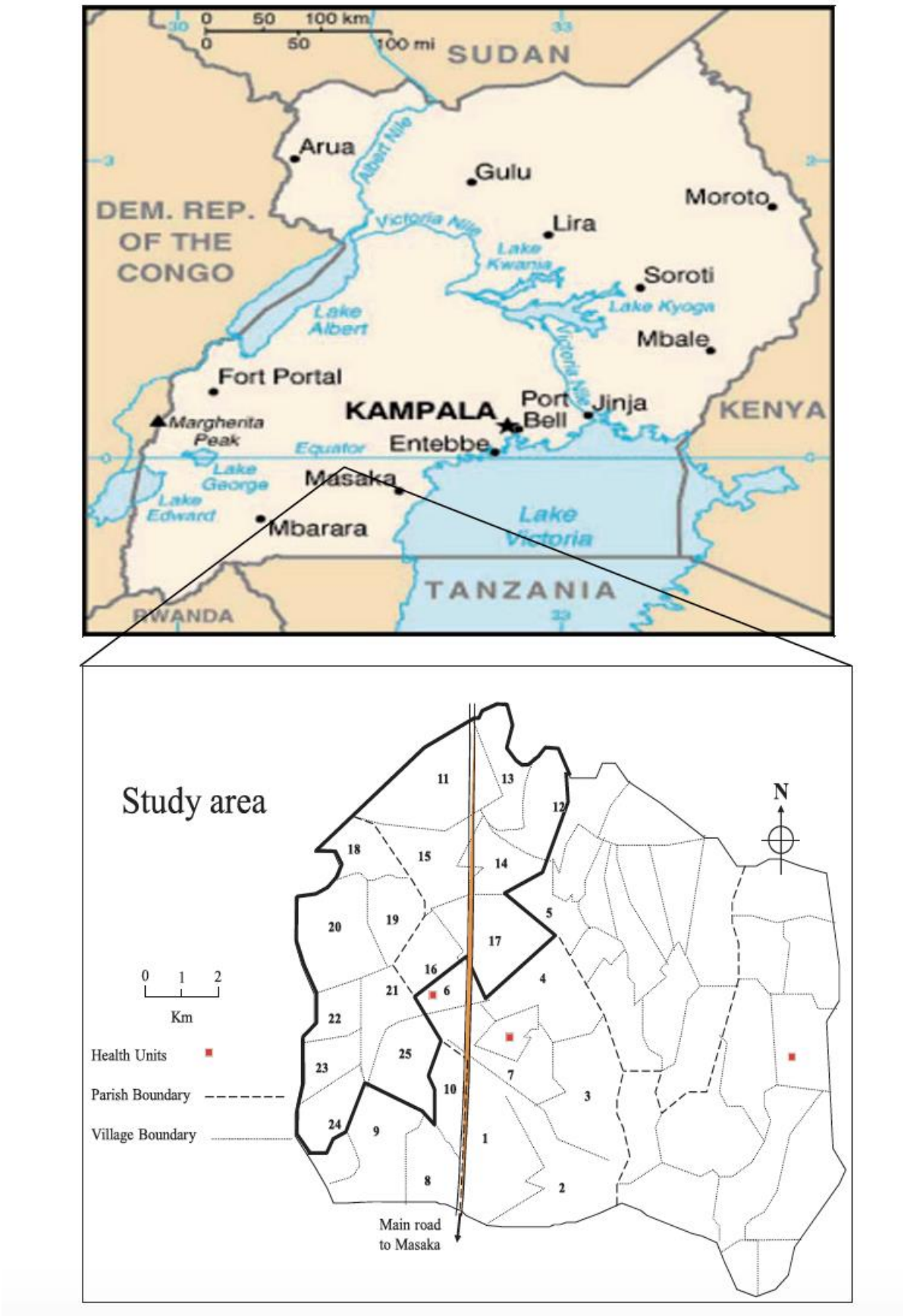

Figure 1: Map showing the location of the General Population Cohort (GPC), from Asiki *et al.,* 2013 [5].

The GPC population has various ethnic groups. The major ethnic group are Baganda (75%), followed by immigrants from Rwanda (16%) and Burundi (3%), lastly, other minor tribes from Uganda and Tanzania make up 6% of the population. The majority of these are Christians; 28% are Muslims [7].

The GPC was established to examine the trends and determinants of the HIV epidemic in rural southwestern Uganda. Originally, annual house-to-house census round surveys were carried out to select participants for the medical survey. Other data collected through the census round survey included participants' location, socio-demographic and household data. During medical surveys, blood, biophysical and lifestyle data were collected to determine disease outcomes and risk factors for selected participants. The process would start with community sensitization activities including local council briefings and village meetings. This would be followed by participant consenting, interviewing using standard questionnaires and phlebotomy at participants' homes. All people who had lived in the village for at least three months or who had the intention of staying were eligible for inclusion. In 2011 at least 84% of the residents had ever participated in the surveys [7-9]. The house to house survey system was changed to a biennial survey conducted at a central hub in each village starting in 2012. The main reasons for the change were to improve on cost-effectiveness, efficient data collection, improved population coverage and increased uptake of HIV test results [4]. To determine KSHV prevalence in this area, we have tested plasma samples for KSHV IgG antibodies, collected at various survey rounds, the latest tested being the 24th (2014/2015) and 25th (2016) surveys. Methods and results from round 24 and 25 analysis were published [10] and are shown in chapter four of this thesis.

In addition to testing plasma samples collected in the 24th and 25th annual surveys [10], we carried out a cross-sectional study to investigate immune correlates and determinates of KSHV viral detection in the GPC. Between July 2017 and November 2017, we recruited 975 KSHV seropositive, HIV seronegative, people aged 2 to 89 years. Participants were

randomly selected from a list of KSHV seropositive, HIV negative people aged 2-89 years, ensuring that one participant was selected from each household and representative numbers were enrolled from each age group. After participants consented, they were counselled and tested for HIV as an eligibility criterion. Only HIV negative people were included in the study. Blood (20ml), saliva and stool samples were collected from enrolled participants aged 10 years and above. Children below 10 years gave 10ml of blood as well as stool and saliva. Stool was tested for helminths using the Kato Katz microscopy methods, EDTA blood was used for malaria diagnosis using rapid diagnostic tests (RDT) (ONE STEP Malaria HRP-II (P.f) and pLDH (Pan) Antigen Rapid Test) and full blood count using a Coulter haematology analyser (Ac.t 5 Diff CP, Beckman Coulter). Peripheral blood mononuclear cells (PBMCs) were isolated from an ACD blood tube and stored for ELISpot and flow cytometry experiments. Plasma from the ACD tubes was used for ELISA and Luminex assays. PBMC and saliva pellets were also used for KSHV viral load quantification using PCR. After sample collection, questionnaires (mainly for demographic information) were filled and transport refund issued to all invited study participants. More details and results from this work are shown in chapters six and seven of this thesis.

- **The Lake Victoria Island Intervention Study on Worms and Allergy-related diseases (LaVIISWA)**

LaVIISWA was a two-arm open cluster randomised trial (ISRCTN47196031) of intensive versus standard antihelminthic treatment. The trial was carried out in Lake Victoria island fishing villages in Koome sub-county, Mukono district, Uganda (Figure 2), between September 2012 to August 2016 [11]. Koome islands consists of 27 villages with a population of approximately 16,000 people and all the villages participated in the trial. These villages are 2-3 hours from Entebbe using a powered canoe. They are well defined and geographically separated with a single administrative committee per village; the administrative committee is generally located near the lake shores [12]. The trial aimed to investigate the effect of helminth treatment on allergy-related outcomes in a *Schistosoma*

mansoni heavily infected area. Standard treatment comprised of a single dose of albendazole (400mg) given twice yearly and a single dose of praziquantel (40mg/Kg) given annually, while the intensive treatment was a triple dose of albendazole (400mg) given three times annually and a single dose of praziquantel (40mg/Kg), given four times yearly. Twenty-six villages were randomised to receive either intensive or standard helminth treatment at a ratio of 1:1. The remaining village was used for the pilot study. The unit of randomisation was the villages as opposed to individuals or households. This was mainly to prevent dilution of the treatment effects by neighbours who fail to take the treatment. It was also useful to attain community level reduction in transmission. In each village, all households were eligible for inclusion in the study, but due to a large number of households, a simple random number of 45 households per village was selected to be included in the study. All members of the selected households were eligible for inclusion. Two main surveys were carried out, one at the beginning (baseline) before the treatment intervention and the second after three years of the trial intervention. During these surveys, trial drugs were home delivered. Questionnaires were administered to record household features and socio-demographic characteristics. A general history and examination were performed, HIV counselling and testing was carried out, followed by sample collection (blood, urine and stool) [11-13]. A total of 1310 plasma samples collected at baseline before the trial intervention as well as from 1571 plasma samples, collected after three years of the trial intervention were randomly selected and tested for KSHV IgG antibodies to determine KSHV seropositivity in this area and then its association with *Schistosoma mansoni* infection. More details of this analysis are shown in chapter five of this thesis.

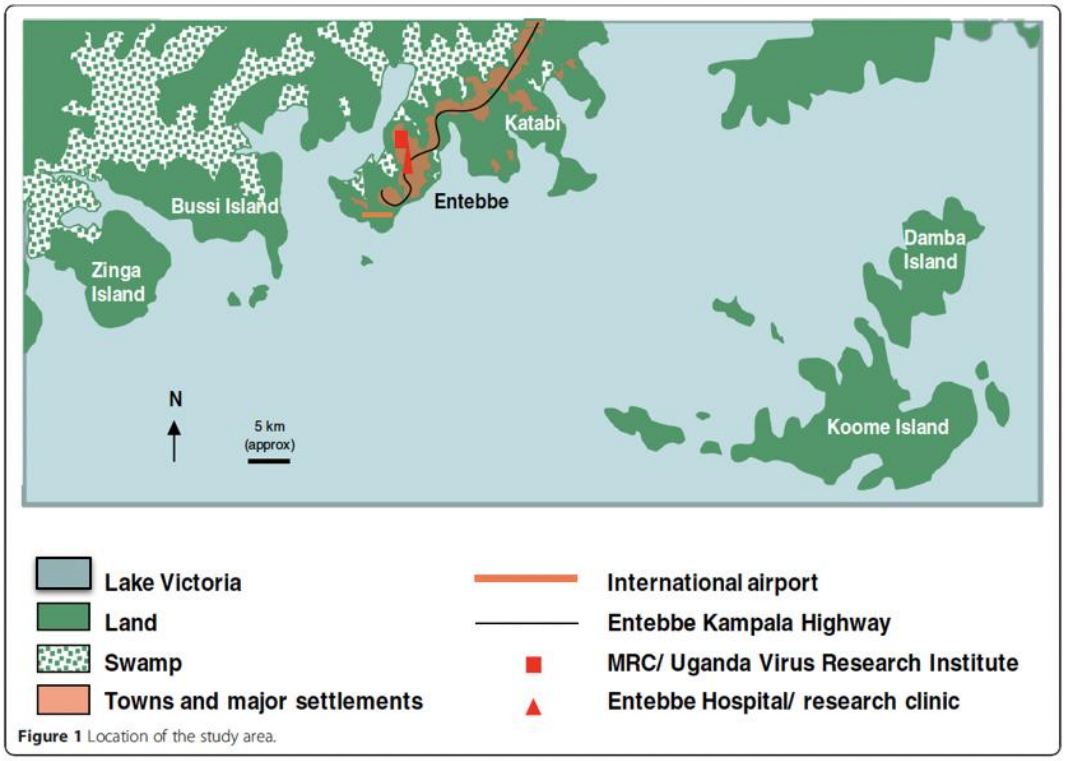

Figure 2: Location of the LaVIISWA area, from Nampijja *et al.,* 2015 [12].

- **The Entebbe Mother and Baby Study (EMaBS)**

The Entebbe Mother and Baby Study (ISRCTN32849447) is a randomised double-blind, placebo control trial of anthelminthic treatment during pregnancy. EMaBS was designed to investigate the effects of helminth treatment during pregnancy on infant responses to vaccines and infectious diseases. Pregnant mothers in their second or third trimester attending antenatal care at Entebbe general hospital were recruited between June 2003 and August 2005. A total of 2507 pregnant women from Entebbe municipality and Katabi sub-county (Figure 3) were enrolled in the study. The inclusion criteria were residence in the study area (Entebbe and Katabi), planning to deliver from Entebbe general hospital and willingness to have an HIV test. Women with haemoglobin below 8g/dL, clinically apparent severe liver disease, diarrhoea with blood in the stool, pregnancy abnormalities and/or a history of adverse reaction to anthelminthic drugs were excluded from the study. The study drugs were a single dose of 400mg of albendazole and 40mg/Kg of praziquantel. After

delivery, the children at age 15 months to five years were also randomised to receive either quarterly albendazole or placebo [13-16]. Demographic and clinical details, blood and stool samples were obtained from the mothers at enrolment and one month after delivery, and from children at ages one, two, three, four, five, six and nine years annually. These samples were stored after use, for future studies. Using these plasma samples, we tested for KSHV antibody responses to determine the age at KSHV seroconversion and its effect on subsequent antibody responses. Results from this analysis were published [17] and are shown in chapter three of this thesis.

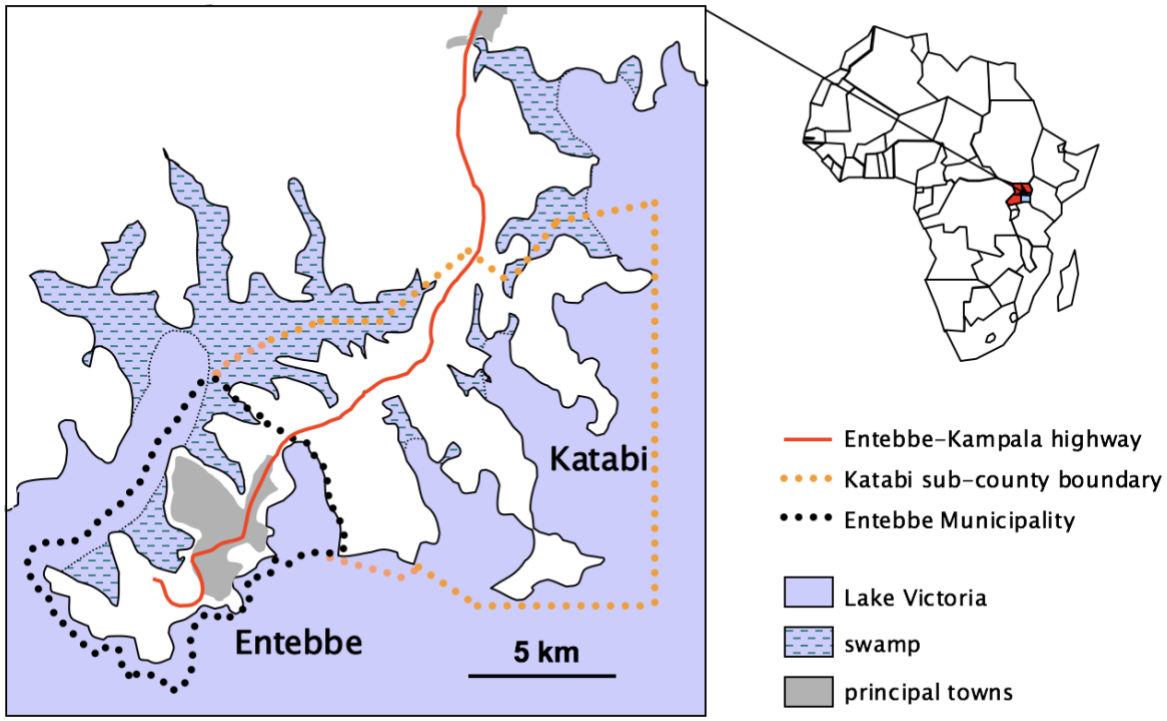

Figure 3: The Entebbe Mother and Baby Study site area. Figure provided by Professor Alison Elliott

Laboratory methods

Samples collected from the three populations were tested for various infections and immunological parameters. Blood collected in ACD vacutainer tubes was centrifuged at 1000xg for 5 minutes. Plasma was removed, stored at -80°C and peripheral blood mononuclear cells (PBMCs) separated. The remaining blood in the ACD tubes (after plasma

removal) was diluted 1:1 with RPMI (5mL L-glutamine, 5mL pen-strep and 12.5mL HEPES buffer in 500mL of RPMI 1640). Ficoll-Paque (15.5mL) was added to a 50ml Leucosep tube (ref:227290, Greiner Bio-one) and centrifuged at 1000xg for 1 minute to get the Ficoll below the porous filter disc. Diluted blood was transferred into prepared Leucosep tubes and centrifuged at 1000xg for 15 minutes without breaks. A white cloudy band of PBMCs layer was transferred into a sterile 50mL falcon tube and the volume topped up to 45mL with RPMI. These PBMCs were centrifuged at 650xg for 7 minutes. Thereafter the supernatant was poured off and the cells re-suspended in 5mL of ACK lysing solution. This was followed by a 10 minutes incubation at room temperature and addition of 15mL of RPMI after the incubation. Centrifuging at 650xg for 7 minutes followed, and the cells were re-suspended in 5mL of RPMI for cell counting. Cells were counted using an automated cell counter (cellometer Vision Trio Nexcellon bioscience). Two million cells were deducted and centrifuged at 13000xg for 10 minutes followed by removing off the supernatant and storage of the pellet at -80°C. The remaining viable cells were dissolved in freezing media put in cryogenic vials and transferred to -80°C freezer in precooled stratacooler boxes. The cryogenic vials containing the cells were then transferred to liquid nitrogen for long-term storage after the overnight storage in the freezer.

Study participants were asked to rinse their mouths with 5mL of Listerine mouth wash, thereafter spitting Listerine and saliva into a 50mL falcon tube. One mL aliquots of saliva were transferred into 1.5 ml tubes, centrifuged at 13,000xg for 10 minutes. The supernatant was removed and the pellet stored at -80°C for later use.

Plasma samples were tested for anti-KSHV antibody responses using enzyme-linked immunosorbent assay (ELISA) and Luminex assays. Anti-*Schistosoma mansoni* antibody concentrations were also measured using ELISA. KSHV specific IFN-γ responses were determined from PBMCs using enzyme-linked immunospot (ELISpot) assays. PBMCs and saliva samples were tested for KSHV viral load using real-time polymerase chain reaction

(PCR) assays. Helminth infections status was ascertained using microscopy and real-time PCR assays. KSHV specific assays were developed at the Viral Oncology Section/National Cancer Institute, NIH, USA, in collaboration with Denise Whitby and were transferred to the MRC/UVRI and LSHTM Uganda Research Unit, where they are well established. Details of these assays are shown below.

- **KSHV ELISA**

To determine KSHV serostatus, plasma samples from the GPC and LaVIISWA were tested for anti-KSHV IgG Antibodies to KSHV-encoded K8.1 and latently associated nuclear antigen (LANA)/ORF73 recombinant proteins (obtained from Denise Whitby, Viral Oncology Section/National Cancer Institute, NIH, USA) to determine KSHV serostatus following the procedure below. DynexImmulon 4 HBX 96 well plates (D17506, Fisher catalogue number: NC9939836) were coated with 100μl of K8.1 and ORF73 recombinant proteins at a dilution of 1:5,000 for each protein. K8.1 protein was diluted in 0.05M carbonate/bicarbonate buffer, pH 10 and ORF73 in 1x phosphate buffered saline (PBS). The plates were sealed using Nunc plate sealers and incubated in a fridge (4˚C) overnight. After the overnight incubation, plates were washed three times with 350μl of wash solution (1x PBS, 0.05% Tween-20) per well, using an automated plate washer (BioTek ELx405). They were then inverted and tapped dry on paper towels. A volume of 270uL assay buffer (2.5% bovine serum albumin-BSA (Sigma Chemical, catalogue number: A-7284) plus 2.5 % normal donor goat serum (Equitech-Bio catalogue number SG-0500) and 0.005 % tween 20 as well as 0.005 % Triton X-100 in 1x PBS) was added to each plate as a blocking agent, sealed with a plate sealer, and incubated for 3 hours at 37˚C and stored at -80C. To run the assay, plates were thawed and washed three times with 350ul of wash solution prior to adding samples. Plasma samples and controls diluted 1:20 for K8.1 and 1:100 for LANA in assay buffer were added to each plate in a volume of 100μl. Plates were sealed and incubated in a 37°c incubator for 90 minutes. Each plate contained 88 samples, negative and positive controls (each in triplicate) and a blank (assay buffer) in duplicate. After the incubation, plates were washed

five times with wash buffer, tapped dry on paper towels, and 100µl of goat anti-human IgG-Alkaline phosphatase labelled conjugate (KPL catalogue number 4751-1002) at a dilution of 1:5,000 in assay buffer was added per well. Plates were then sealed and incubated at 37°C for 30 minutes. Following the 30 minutes incubation, plates were washed 5 times, tapped dry on the paper towel, and 100µl of 1-step p-nitrophenyl phosphate (PNPP) substrate solution was added per well. They were then developed in the dark at room temperature for 30 minutes for LANA and 25 minutes for K8.1. Plates were then immediately read using a microtiter plate reader (BioTek ELx808) at a wavelength of 405nm. Optical densities (ODs) were obtained. The ODs of the blank wells were used for background subtraction for each sample and control. The positive and negative controls were used for quality control, to determine if a plate has passed. The negative controls were also used to calculate a cut-off value for each plate. The cut-off value for each plate was the average background subtracted OD of the three negative control triplicates plus a constant value of 0.75 (for K8.1) or 0.35 (for LANA). This procedure has been reported previously [18].

- **KSHV Luminex**

The ELISA assay is limited by a narrow dynamic range, a high sample volume and a single antigens testing. To overcome these limitations, samples from the GPC and EMaBS were tested for anti-KSHV IgG antibody levels using a bead-based multiplex assay. The procedure is described below.

Bead coupling

Eight recombinant KSHV-encoded proteins (OFR73, K8.1, ORF65, ORF38, ORF61, K5, K10.5 and ORF19) were coupled to fluorescent magnetic microspores/beads (Megaplex Microsphores, Luminex Corp, Austin, USA) individually, using the following procedure. A volume of 200uL MagPlex Microspheres, containing $2.5x10^6$ beads, was resuspended (vortexed for 20s and sonicated for 20s) and washed using 100µl of nuclease-free water. After removal of the water using a MACSiMAG apparatus, the beads were resuspended in

80µl of activation buffer (0.1M NaH2PO4 in PBS at a PH of 6) and 10µl of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (10mg EDC in 200µl of nuclease-free water) and 10µl of *N*-hydroxysulfosuccinimide (Sulfo-NHS) (10mg Silfo-NHS in 200µl of nuclease-free water) for activation. After 20 minutes incubation, the activation buffer was removed and the beads resuspended in 250µl of coupling buffer (0.05M MES in PBS) followed by removal of the buffer, this step was repeated twice. After washing with coupling buffer, the beads were then resuspended in 100µl of coupling buffer followed by addition of KSHV proteins (at a concentration of 40µg per 2.5 million beads) to each unique bead set. The volume was then adjusted to 500µl using coupling buffer. After a 2hour incubation at room temperature in the dark with gentle mixing, the coupled beads were then resuspended in 500µl of PBS-TBN (1% BSA, 0.05% Sodium Azide, 0.002% Tween20 in PBS) after removal of the previous solution/supernatant. This was followed by resuspension into 1000µl of PSB-TBN after removal of the previous 500µl. The 1000µl supernatant was removed and coupled beads resuspended into 1000µl of PBS-BN (1% BSA and 0.05% sodium azide in PBS). The coupled beads were then counted using a haemocytometer and the bead concentration recorded. The coupled beads were then stored at 4^{o}C.

Bead testing

The coupled beads were then tested using KSHV positive and negative controls before running the actual samples, following a similar procedure as the one used to run the actual samples but varying the dilution of both the controls and detection antibody.

Assay procedure

The coupled beads from each ORF were combined to make a multiplexed bead mixture by pipetting 2,000 beads per well of each bead set coupled to a particular antigen and topping up the volume to make 95µl per well with assay buffer. Using cell culture plates (Costar, Corning) that have been prewashed with assay buffer (1% BSA in PBS), 95µl of bead mixture were added and 5µl of a prediluted (1/10) sample (plasma) with assay buffer added

to make a final sample dilution of 1/200. The plates were sealed and wrapped in aluminium foil followed by agitation for 60 minutes. They were then washed with assay buffer twice using a magnetic plate washer. Followed by the addition of 100µl per well of 0.5µg/ml of the detection antibody (goat $F(ab^{l})_2$ anti-human IgG(y) R-PE conjugate), sealed and wrapped in aluminium foil and incubated for 30 minutes with gentle shaking. After incubation, the plates were washed twice and 100µl per well of assay buffer added, then Median Fluorescence Intensities (MFI) were determined by reading the plate using a Bioplex200 Luminex machine. This procedure was followed as reported previously [19].

- **KSHV specific ELISpot**

Stored PBMCs from 40 participants from the GPC were thawed and tested for KSHV specific cell-mediated IFN-γ responses using ELISpot assays. PBMCs stored in liquid nitrogen were retrieved and immediately transferred to a water bath pre-warmed to 37^0C. With only a small pellet left, the vials with PBMCs were transferred to a class II safety cabinet. Using Pasteur pipettes, cells were transferred to falcon tubes containing 9mL of R-10 (10% foetal bovine serum (FBS) in RPMI) containing benzonase nuclease. This was followed by centrifugation at 350rcf for 5 minutes. After centrifugation, the supernatant was poured off and 5ml of AIM-V media added to the cell pellet. Cells were then resuspended and counted using a light microscope. Thereafter AIM-V solution was topped up to ensure that the cells are resuspended in 100µl per 150,000 PBMCs. These cells were then temporally stored in the 37ºC 5% CO_2 incubator as the ELISpot plates were being prepared. The MABTECH Human IFN-γ ELISpot kit (Code: 3420-2APT-10) was used for the assay, with a few alterations to the manufacturer's protocol. The 96 well ELISPOT plates coated with the IFN-γ capture antibody from the kit were removed from the fridge and washed manually 5 times with 200µl of 1xPBS per well. Thereafter, thawed cells which were temporally stored in the incubator were added to the plates in a volume of 100µl containing 150,000 cells per well. The plates were covered with the lid and wrapped in aluminium foil

and transferred to a 5% CO_2 37ºC incubator for resting. The cells were rested for 24 hours. To stimulate the cells, overlapping peptides across the KSHV proteome were generated and 84ORF peptide pools created. Eighty-one of which corresponded to a single ORF and the three to ORF64. The peptide pools were supplied by the Viral Oncology Section/National Cancer Institute, NIH, USA. Using these peptide pools, working concentrations of 5μg/ml were prepared in 96 well culture plates using AIM-V media. After the 24 hour resting period, 100μl per well of the peptide pools was added to the cells to stimulate them. Anti-CD3 and a pool of peptides from flu, CMV and EBV were added as positive controls in triplicate. Media and a non-human antigen, Simian immunodeficiency virus (SIV) peptides were also added as negative controls in triplicate. The cells with the stimulants were then incubated at 37ºC for 46-48 hours. Following the 48-hour stimulation, cells were washed 5 times with 200μl of PBS per well and 100μl of anti-human IFNγ IgG alkaline phosphatase conjugated (Code: 7-B6-ALP) added at a dilution of 1/6 in PBS + 0.5% FBS. The plates were incubated at room temperature (25^0C) for 2 hours. After the incubation, the plates were washed 5 times with 200μl of 1xPBS per well and 100μl of filtered 5-bromo-4-chromo-3-indolyl-phosphate (BCIP)/nitroblue tetrazolium (NBT)-plus substrate (Code: 3650-10) added per well. Thereafter plates were incubated at room temperature for 7 minutes and the reaction was stopped by washing the plate with running tap water. These plates were dried in the dark overnight, thereafter the spots were counted using an ELISPOT reader (AID ELISPOT Reader HR model: ELHR010307005). Results from the ELISpot assay are presented in chapter seven of this thesis.

- **KSHV quantitative real-time polymerase chain reaction (PCR)**

KSHV viral load was quantified from peripheral blood mononuclear cells (PBMCs) and saliva collected from 877 KSHV seropositive individuals from the GPC. After PBMC isolation using the ficoll density gradient method, two million cells were removed, spun at 13,000rcf for 10minutes to form cell pellets. The supernatants were poured off and the cell pellets stored

at -80^{o}C. Similarly, 1mL aliquots of saliva were spun at 13,000rcf for 10 minutes to form saliva pellets. The saliva supernatants were aliquoted into new storage tubes. Both the saliva pellets and supernatants were stored at -80^{o}C until use. The PBMC and saliva pellets were retrieved from the -80^{o}C freezer, thawed and genomic DNA extracted using a QIAamp blood kit (Qiagen, Valencia, CA), following the manufacturer's instructions. KSHV viral load was quantified using real-time PCR. KSHV DNA was detected using primers (K6 forward primer K6-10F 5'-CGCCTAATAGCTGCTGCTACGG-3', K6 reverse primer K6-10R 5'-TGCATCAGCTGCCTAACCCAG-3') and a probe (K6 probe p-K6-10 5'-R-CACCCACCGCCCGTCCAAATTC-Q-3') previously reported to be specific to the K6 gene region [20]. Additionally, the number of cellular equivalents were determined using a quantitative assay specific to human endogenous retrovirus 3 (ERV-3), which is present in two copies per genomic cell, using these primers (ERV-3 Forward primer PHP10-F 5'-CATGGGAAGCAAGGGAACTAATG' ERV-3 Reverse primer PHP10-R 5'-CCCAGCGAGCAATACAGAATTT-3') and a probe (ERV-3 Probe PHP-P505 5'-R-TCTTCCCTCGAACCTGCACCATCAAGTCA-Q-3'). The ERV-3 assay was run on only PBMC DNA. To quantify both ERV-3 and KSHV DNA, seven two-fold serial dilutions of K6 and ERV-3 were made from clone stocks (starting with $1x10^{6}$ dilution to $1x10^{-1}$) to form a standard curve on every plate. The clone stocks were prepared and supplied by the Viral Oncology Section/National Cancer Institute, NIH, USA. ERV-3 was cloned into Bluescript II KS vector (Stratagene, La Jolla, CA, USA) KSHV K6 cloned using PCR Topo II vector, Topo TA Cloning kit, Invitrogen, K 4600-40. Primers (500ul each), probes (500ul), nuclease-free water (5.5ml) and universal master mix (Applied Biosystems, Foster City CA) (12.5ml) were mixed together to form the working solution master mix. Thereafter 10μl of the standards, sample and controls each, were added to a 96 well PCR plate (ThermoFisher catalogue number 7306737) per well, in triplicate, and 40ul of the working solution master mix added to each well. This was followed by plate sealing and DNA amplification using an ABI ViiA7 machine. All samples were tested in triplicate for both assays and the estimated copy number for each individual reaction was averaged. The KSHV viral load was determined in

PBMC DNA by calculating the viral DNA copies per million cells. Saliva viral copies were reported as copies per millilitre. Any sample that was quantifiable by the ERV-3 assay but not positive in all three reactions in the KSHV K6 assay was designated as positive but not quantifiable. The lower limit of detection for the ERV-3 assay has been previously determined as 10 copies while the KSHV K6 assay has a lower limit of sensitivity of 1 copy. This procedure has been reported elsewhere [21-23].

In order to prevent contamination, qPCR reagents, sample processing, DNA extractions, and qPCR setup were conducted in dedicated laboratory areas. Internal no template controls (NTC) were included on each plate, in triplicate, to assess cross-contamination. Assay quality controls including NTC, assay controls, and sample triplicate % coefficient of variation were used to validate data. Importantly, the individual dilution parameters of the assay standard curve were assessed after each run to ensure performance within expected ranges. Any assay which had failures of assay controls or standard curves were repeated.

- **Stool real-time polymerase chain reaction (PCR)**

Multiplex real-time stool PCR was performed on LaVIISWA samples to detect *Schistosoma mansoni*, *Strongyloides stercoralis* and *Necator americanus*, following published protocols [24, 25]. A PCR assay was used as this is reportedly more sensitive than microscopy [24, 25] and *Strongyloides stercoralis* could not be detected by microscopy. Additionally, the two hookworm species could only be identified by PCR. DNA from stool samples was extracted using QIAamp DNA mini kits (catalogue number: 51306) following the manufacturer's instructions. Primers, probes, magnesium chloride, Hotstart Taq Master mix, bovine serum albumin (BSA) and nuclease-free water were mixed together to make a working solution of master mix using the following concentrations: magnesium chloride 3.5mM, BSA 0.1mg/ml, forward primer *S. stercolaris* 100nM, reverse primer *S. stercolaris* 100nM, probe *S. stercolaris* 80nM, forward primer *N. amaricanus* 200nM, reverse primer *N. amaricanus* 200nM, probe *N. amaricanus* 80nM, forward primer *S mansoni* 100mM, reverse primer *S mansoni* 100nM, probe *S mansoni* 80nM, forward primer Phocine Herpes Virus (PhHV)

100nM, reverse primer PhHV 100nM, probe PhHV 80nM, Hotstart Taq Master mix 54.3%, nuclease-free water 21%. A volume of 23μl of this working solution master mix and 1μl of PhHV plus either a sample or control DNA volume of 1μl were added to each well of the FAST MicroAmp 96-well reaction PCR plates. The plates were sealed and loaded into a PCR machine (ABI 7500) for amplification using already established protocols. PhHV DNA was used as an internal control. Primer and probe sequences used were *Necator americanus* (Na58F 5'-CTGTTTGTCGAACGGTACTTGC-3', Na158R 5'-ATAACAGCGTGCACATGTTGC-3' Na81MGB, FAM-5'-CTGTACTACGCATTGTATAC-3'-XS), *Schistosoma mansoni* (Ssp48F 5'-GGTCTAGATGACTTGATYGAGATGCT-3', Ssp124R 5'-TCCCGAGCGYGTATAATGTCATTA-3', Ssp78T-RT Texas Red-5'-TGGGTTGTGCTCGAGTCGTGGC-3'-BHQ2), *Strongyloides stercoralis* (Stro18S-1530F 5'-GAATTCCAAGTAAACGTAAGTCATTAGC-3', Stro18S-1630R 5'-TGCCTCTGGATATTGCTCAGTTC-3', Stro18S-1586T NED-5'-ACACACCGGCCGTCGCTGC-3'-BHQ2) and Phocin Herpes Virus (PhHV-267 s5'-GGGCGAATCACAGATTGAATC -3', PhHV-337as 5'-GCGGTTCCAAACGTACCAA -3', PhHV-305tq Cy5 -5'-TTTTTATGTGTCCGCCACCATCTGGATC-3'-BHQ2). Samples with positive amplifications were noted as positive and the amount of helminths DNA was derived from the Ct (cycle threshold) values.

- **Kato Katz (stool microscopy)**

A single stool sample was provided by each participant from the GPC and LaVIISWA. This stool sample was analysed for helminths (*Schistosoma mansoni*, *Ascaris lumbricoides*, *Tichuris trichiura*, *Trichostrongylus spp* and hookworm) using the Kato Katz method following the kit manufacturer's instructions. A small sample of stool was sieved using nylon gauze. Using a template an equal amount of stool (41.7mg) was prepared per slide. Glycerol was used to clean away the debris material and malachite green was used to stain the background for visibility of the eggs. Two slides were prepared and independently examined for worm eggs using a light microscope by two laboratory technologists independently. The

number of eggs per gram of stool was recorded. Light, moderate and heavy infections were categorised following kit manufacturer's instructions. Details of this procedure have been reported elsewhere [26, 27]. Samples from the GPC were kept at 4^{o}C overnight and examined the day after, while samples from LaVIISWA were analysed immediately after collection.

Ethical approvals

Work presented in this thesis was approved by the Uganda Virus Research Institute-Research and Ethics Committee (UVRI-REC) reference numbers: GC/127/16/09/566 and GC/127/17/04/317, the Uganda National Council for Science and Technology (UNCST) reference numbers: HS2123 and HS1183 and the London School of Hygiene & Tropical Medicine (LSHTM) Ethics Committee reference numbers: 11881 and 9917-9. All participants aged 18 years and above provided written informed consent for participation in the studies and storage of their samples for future use. Children below 18 years were consented for by their parents or guardians. In addition, written informed assent was given by participants aged 8 to 17 years. Approval letters are presented in Appendix 1.

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Results chapters

Results from the PhD research are presented in five chapters (from chapter three to chapter seven) of the thesis. The first three chapters focus on risk factors for KSHV seropositivity and/or antibody levels. Chapter six shows patterns of KSHV DNA detection and associated risk factors. Chapter six also shows the relationship between KSHV antibody levels and viral DNA detection. The final result chapter shows IFN-γ cellular responses to KSHV peptide pools.

Chapter Three: Age of infection and KSHV antibody levels in Uganda

Preamble

This PhD project focused on understanding factors that may cause high levels of transmission of KSHV in Uganda. Transmission of KSHV in endemic areas occurs during childhood. The consequences of early infection for the natural history of the virus and the risk of KSHV-related disease have not been fully explored. Using samples from an existing birth cohort, we determined the age at which children acquired KSHV infection by measuring antibody levels to KSHV. We then determined the association between the age of infection with KSHV and subsequent KSHV antibody levels. The results showed a very early age of infection and that early infection was associated with higher subsequent antibody levels. These high antibody levels were sustained over several years and may remain high for life. High antibody levels to KSHV have been correlated with KS disease risk. Therefore, these results may imply a role of early infection in relation to increased subsequent risk of KS in Africa.

Results from the antibody levels to K8.1 and ORF73 were published in the Pediatric Infectious Disease Journal. Antibody levels to other KSHV antigens including K10.5, ORF19, ORF61, ORF38, K5 and ORF65 are shown in Chapter Three - additional results.

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SECTION A – Student Details

Student	Angela Nalwoga
Principal Supervisor	Stephen Cose
Thesis Title	Determinants of Kaposi's sarcoma associated herpesvirus seropositivity, viral DNA detection and cellular immune responses in Uganda

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	The Pediatric Infectious Disease Journal		
When was the work published?	August 2018		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	NO		
Have you retained the copyright for the work?*	No	Was the work subject to academic peer review?	**Yes**

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Student Signature: [redacted] **Date:** 20th May 2019

Supervisor Signature: [redacted] Date: 21/05/2019

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AGE OF INFECTION WITH KAPOSI SARCOMA–ASSOCIATED HERPESVIRUS AND SUBSEQUENT ANTIBODY VALUES AMONG CHILDREN IN UGANDA

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Abstract: We investigated associations between Kaposi sarcoma–associated herpesvirus (KSHV) seroconversion age and KSHV antibody values in Ugandan children. Every annual delay in KSHV seroconversion age was associated with a reduction of 19% ($P < 0.0001$) in K8.1 and 27% ($P < 0.0001$) in ORF73 antibody values at 6 years of age. Early infection may be an important risk factor for KSHV pathogenesis and viral shedding in saliva, leading to transmission.

Key Words: Kaposi sarcoma–associated herpesvirus, seroconversion age, antibody values, Uganda

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Transmission of Kaposi sarcoma–associated herpesvirus (KSHV) is mainly through salivary exchange and can occur in childhood in endemic regions[1]; KSHV prevalence increases with age.[2] The impact of age of infection with KSHV on the pathogenesis and control of KSHV has not been investigated.

Early infection with Epstein-Barr virus (EBV), another gammaherpesvirus closely related to KSHV, is associated with higher subsequent viral load.[3] High viral capsid antigen antibody titers and EBV viral load have been associated with risk of Burkitt lymphoma,[4] the most common childhood malignancy in equatorial Africa, linked to both EBV and malaria.[5]

High antibody titers to KSHV are an important predictors of risk of Kaposi's sarcoma (KS) disease[6]; they are also a marker of KSHV reactivation.[7] This study was designed to determine the association between the age at which children from Uganda become KSHV seropositive (KSHV seroconversion age) and subsequent KSHV-specific immunoglobulin G (IgG) antibody values.

METHODS

Study Population

Samples collected from children enrolled in the Entebbe Mother and Baby Study were tested for KSHV IgG antibody responses retrospectively. Entebbe Mother and Baby Study was initiated as a randomized controlled trial, designed to investigate the impact of helminth treatment during pregnancy on childhood responses to vaccines and infectious diseases. The trial protocol and results have been described elsewhere.[8] A total of 2507 pregnant women from Entebbe, Uganda, a semiurban area, were recruited and their children have been followed from birth. Blood and other samples have been collected annually and stored.

Ethical Approval

This study was approved by the Uganda Virus Research Institute—Research and Ethics Committee, the Uganda National Council for Science and Technology and the London School of Hygiene & Tropical Medicine. Informed consent was obtained from study participants' parents or guardians.

KSHV Serologic Testing

Plasma samples collected at 6 years of age (annual 6) were tested for KSHV IgG antibodies to identify KSHV seropositive children. KSHV seropositivity was defined by seropositivity to either ORF73 or K8.1 antigen. Any seropositive child was then tested at age 5, and so on retrospectively, until a seronegative specimen was identified. To determine if the effect of age at infection on subsequent antibody values is sustained for a longer time period, we then tested the available plasma samples at age 9 from the

TABLE 1. Association Between Age of KSHV Seroconversion, Sex, Age and KSHV Antibody Levels Among Children From Uganda

	K8.1				ORF73			
AI: Antibody Levels at Age 6 yr From 158 Children								
	Crude GMR (95% CI)	*P*	Adjusted* GMR (95% CI)	*P*	Crude GMR (95% CI)	*P*	Adjusted* GMR (95% CI)	P
Seroconversion age	0.81 (0.72–0.90)	<0.0001	0.81 (0.73–0.91)	<0.0001	0.73 (0.64–0.83)	<0.0001	0.73 (0.64–0.83)	<0.0001
AII: Antibody Levels at Age 6 yr From 100 Children With All Consecutive Samples								
	K8.1				ORF73			
	Crude GMR (95% CI)	*P*	Adjusted* GMR (95% CI)	*P*	Crude GMR (95% CI)	*P*	Adjusted* GMR (95% CI)	*P*
Seroconversion age	0.78 (0.69–0.89)	<0.0001	0.80 (0.70–0.37)	0.001	0.69 (0.59–0.81)	<0.0001	0.68 (0.58–0.80)	<0.0001
BI: Antibody Levels at Age 9 yr From 158 Children								
	K8.1				ORF73			
	Crude GMR (95% CI)	*P*	Adjusted* GMR (95% CI)	*P*	Crude GMR (95% CI)	*P*	Adjusted* GMR (95% CI)	*P*
Seroconversion age	0.67 (0.57–0.79)	<0.0001	0.67 (0.57–0.79)	<0.0001	0.75 (0.66–0.85)	<0.0001	0.75 (0.66–0.85)	<0.0001
BII: Antibody Levels at Age 9 yr From 100 Children With All Consecutive Samples								
	K8.1				ORF73			
	Crude GMR (95% CI)	*P*	Adjusted* GMR (95% CI)	*P*	Crude GMR (95% CI)	*P*	Adjusted* GMR (95% CI)	*P*
Seroconversion age	0.96 (0.78–1.18)	0.691	0.95 (0.77–1.17)	0.620	1.03 (0.88–1.19)	0.740	1.03 (0.88–1.20)	0.737
CI: Antibody Levels at Ages 1, 2, 3, 4, 5, 6 and 9 yr From 158 Children								
	K8.1				ORF73			
	Crude GMR (95% CI)	*P*	Adjusted† GMR (95% CI)	*P*	Crude GMR (95% CI)	*P*	Adjusted GMR† (95% CI)	*P*
Seroconversion age	0.72 (0.66–0.78)	<0.0001	0.60 (0.55–0.65)	<0.0001	0.71 (0.65–0.77)	<0.0001	0.59 (0.53–0.64)	<0.0001
Age	0.93 (0.85–0.99)	0.018	1.70 (1.57–1.85)	<0.0001	0.82 (0.76–0.88)	<0.0001	1.65 (1.53–1.77)	<0.0001
CII: Antibody Levels at Ages 1, 2, 3, 4, 5, 6 and 9 yr From 100 Children With All Consecutive Samples								
	K8.1				ORF73			
	Crude GMR (95% CI)	*P*	Adjusted† GMR (95% CI)	*P*	Crude GMR (95% CI)	*P*	Adjusted GMR† (95% CI)	*P*
Seroconversion age	0.80 (0.73–0.87)	<0.0001	0.73 (0.67–0.80)	<0.0001	0.78 (0.72–0.85)	<0.0001	0.68 (0.62–0.75)	<0.0001
Age	1.09 (1.02–1.17)	0.008	1.20 (1.12–1.28)	<0.0001	1.22 (1.15–1.03)	<0.0001	1.29 (1.22–1.37)	<0.0001

*Adjusted for sex and seroconversion age.

†Adjusted for age, sex and seroconversion age.

AI and AII show the association between age of KSHV seroconversion, sex and KSHV antibody levels at age 6. BI and BII show the association between age of KSHV seroconversion, sex and KSHV antibody levels at age 9. CI and CII show the association between age of KSHV seroconversion, sex, age and KSHV antibody levels among children at ages 1, 2, 3, 4, 5, 6 and 9 combined from Uganda. AI and CII show results from 100 children with all 6 consecutive samples plus 58 children missing 1–4 samples. AII and CII show results from 100 children with all 6 consecutive samples. BI shows results from 130 children with samples available at age 9. BII shows results from 79 children with available samples at age 9, and have all previous consecutive samples. GMR and 95% CI obtained by calculating the $\log_{10}$ exponent of the regression coefficient and the 95% CI, respectively. Regression coefficient, 95% CI and *P* value were obtained using linear regression after $\log_{10}$ transformation of K8.1- and ORF73-specific total IgG MFI. MFI was obtained using the bead assay. K8.1 and ORF73 IgG antibody responses were analyzed using separate linear regression models. Seroconversion age is the age at which the children became KSHV seropositive. Age is the number of years the children had lived by the time of sample collection.

children who were seropositive at age 6 for IgG antibody responses to KSHV. The estimated age of KSHV seroconversion was defined as the midpoint between the last seronegative and the first seropositive specimen. Because of missing specimens, these samples were not always from consecutive years.

An in-house multiplexed bead assay was used to measure KSHV-specific IgG antibody responses as previously described.[9] This assay has a wider dynamic range than an enzyme-linked immunosorbent assay which is an important advantage when comparing antibody values. ORF73 and K8.1 recombinant proteins were coupled to fluorescent magnetic beads (Biorad, Hercules, CA) according to the manufacturer's protocol. Coupled beads were mixed with plasma samples at a sample dilution of 1/200 and a bead concentration of 2000 beads per well in assay/wash buffer (1% bovine serum albumin in 1 × phosphate buffered saline), to make a total volume of 100 µ per well. The mixture was incubated for an hour under gentle agitation and washed with wash buffer thereafter. Detection antibody of 100 µ of 0.5µL/mL [goat $F(ab')^2$ antihuman IgG R-phycoerythrin (R-PE) conjugate] was added and incubated for 30 minutes under gentle agitation. After washing, 100 µ of assay

buffer was added per well, agitated for 2 minutes and the plate read using a Bioplex200 machine (Luminexcorp, Austin, TX) to obtain the median fluorescence intensities (MFIs). Each plate contained 3 negative and 3 positive control wells plus 2 blank wells. The cutoff MFI values for OFR73 and K8.1 were 968 and 741, respectively, plus the mean values of the negative control per plate.

Statistical Analysis

Data analysis was performed using Stata-13 software (STATA 13.0; Statacorp, College Station, TX). Antibody values (measured as MFIs) were $\log_{10}$ transformed. Linear regression was used to examine the relationship between KSHV IgG antibody responses at 6 and 9 years of age and seroconversion age while adjusting for sex. To investigate the association between seroconversion age and antibody values (KSHV IgG antibodies) at all timepoints/ages (1, 2, 3, 4, 5, 6 and 9), we used mixed models with random effects. Random-effects modeling provided associations that were independent of the duration of infection and accounted for correlation of results from the same child at different time points. Geometric mean ratios (GMRs) and their 95% confidence intervals (CIs) were obtained by calculating the $\log_{10}$ exponent of the regression coefficients and their 95% CI, respectively. Antibodies to K8.1 and ORF73 were analyzed using separate regression models.

RESULTS

Age at KSHV Seroconversion

The number of children who were KSHV seropositive at 6 years of age was 176/535 (33%), 128/535 (24%) were seropositive to K8.1 and 165/535 (31%) were seropositive to ORF73 proteins. The number of children with all 6 consecutive samples was 100/176. Therefore, 76/176 children had at least 1 missing sample, 39, 11, 5, 3 and 18 had 1, 2, 3, 4 and 5 missing samples, respectively. The 18 children with 5 consecutive missing samples were excluded from the analysis, leaving a total of 158 children for analysis. Results from 100 participants with all 6 consecutive samples (Table 1, AII and CII) were comparable to those from 158 participants (Table 1, AI and CI) at 6 years of age and at all ages combined. At 9 years of age, the available samples from the 100 participants were 79 which might have reduced the power of the study to detect statistically significant differences (Table 1, BII). Among the 158 KSHV seropositive 6-year-old children analyzed, 43, 50, 23, 14, 18 and 10 children were estimated to have seroconverted by ages 6, 5, 4, 3, 2 and 1, respectively. The proportions of seroconverters who were boys at the different seroconversion age bands were 22/43 (51%), 26/50 (52%), 14/23 (61%), 8/14 (57%), 10/18 (56%) and 8/10 (80%) at 6, 5, 4, 3, 2 and 1, respectively. Antibody values increased with age. For every annual increase in age, we observed a 71% ($P < 0.0001$) and 65% ($P < 0.0001$) increase in K8.1 and ORF73 IgG antibody values, respectively (Table 1, CI). On the other hand median antibody values generally decreased with increasing age at primary infection (Fig., Supplemental Digital Content 1, http://links.lww.com/INF/C950).

We investigated the association between age at KSHV seroconversion and antibody values to K8.1 and ORF73 antigens at 6 and 9 years of age using sex-adjusted linear regression modeling. At 6 years of age, antibody values decreased with increasing seroconversion age. For every year of delay in seroconversion age, we observed a 19% decrease in K8.1 antibody values, with an adjusted GMR (aGMR) of 0.81, 95% CI (0.73–0.91), $P = 0.001$ (Table 1, AI). In addition, for every year of delay in seroconversion age, we observed a 27% decrease in ORF73 antibody values, with an aGMR of 0.73, 95% CI (0.64–0.83), $P < 0.0001$ (Table 1, AI).

At 9 years of age, both K8.1 and ORF73 antibody values decreased with increasing seroconversion age. For every year of delay in seroconversion age, we observed a 33% decrease in K8.1 antibody values, with an aGMR of 0.67, 95% CI (0.57–0.79), $P > 0.0001$ (Table 1, BI). Similarly, for every year of delay in seroconversion age, we observed a 25% decrease in ORF73 antibody values, with an aGMR of 0.75, 95% CI (0.66–0.85), $P < 0.0001$ (Table 1, BI).

To determine the effect of age of infection on antibody titers which is independent of duration of infection, we then investigated the association between seroconversion age and antibody values at all ages/timepoints (1, 2, 3, 4, 5, 6 and 9) to K8.1 and ORF73 using random-effects models adjusting for sex and age. Generally, antibody values to both K8.1 and ORF73 decreased with increasing seroconversion age. For every year of delay in seroconversion, we detected a 40% decrease in K8.1 antibody values, aGMR 0.60, 95% CI (0.55–0.65), $P < 0.0001$ (Table 1, CI). Similarly, for every year of delay in seroconversion, we observed a 41% reduction in ORF73 IgG antibody values, aGMR 0.59, 95% CI (0.53–0.64), $P < 0.0001$ (Table 1, CI).

DISCUSSION

In KSHV endemic areas, infection can occur early in life, but the importance of age of infection to subsequent transmission and disease risk has not been investigated before. Antibody responses to KSHV, and in particular, values of antibodies, have been associated with KS development, KSHV reactivation and KSHV transmission.[6,7]. In this study, we have observed very early seroconversions and detected a strong association between age of KSHV seroconversion and subsequent antibody values to both K8.1 and ORF73. The earlier these children seroconverted, the higher their subsequent antibody values to both K8.1 and ORF73 proteins. To our knowledge, this is the first study to look at the effect of age of infection with KSHV on subsequent antibody responses. Antibody responses are a proxy measure of KSHV reactivation.[6] K8.1 is a glycoprotein expressed during the lytic phase of the virus life cycle and ORF73 encodes the latently associated nuclear protein, a structural protein expressed during the latent stage of the virus life cycle. Measurement of other parameters related to disease and transmission risk such as viral load in saliva and in blood in relation to KSHV age of infection would be of great interest.

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MACRO- AND MICROVASCULAR PARAMETERS AFTER TOXIC SHOCK SYNDROME

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Abstract: Whether individuals who had toxic shock syndrome in childhood have differences in macro- and retinal microvascular parameters indicative of increased cardiovascular risk is unknown. We found no evidence of adverse macrovascular changes in 22 toxic shock syndrome participants compared with 60 control participants. Microvascular comparisons showed a reduction in retinal total fractal dimension, which has been associated with cardiovascular risk factors in children.

Key Words: toxic shock syndrome, intima-media thickness, arterial stiffness, microcirculation

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D.P.B., M.C., N.C. conceived and designed the experiment. K.Y.H.C., D.P.B., N.C. and M.C. participated in the acquisition, analysis and interpretation of data. K.Y.H.C. drafted the article. All the authors have read and approved of the final article.

The authors have no conflicts of interest to disclose.

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Inflammation is central to the development of atherosclerosis, and pathogenic lesions in arteries are reported from early childhood onward.[1] Children with acute and chronic inflammatory diseases, including Kawasaki disease, inflammatory bowel disease, psoriasis and juvenile arthritis may have persistent adverse changes in their vascular structure and function, although the implications for later cardiovascular disease risk are largely unknown.[2–4] Toxic shock syndrome (TSS) is a rare, acute, life-threatening toxin-mediated illness causing fulminant vascular inflammation and dysfunction, often resulting in multiorgan failure. It is unknown whether children with TSS have changes in either the macro- or microvasculature years after the acute illness and are at increased long-term cardiovascular risk.

We aimed to investigate whether participants with past TSS have quantitative subclinical changes in macro- and retinal microvascular parameters indicative of a possible increased in cardiovascular risk. The retinal and coronary vasculature share many similarities and respond to common metabolic risk factors.[5] We hypothesized that, compared with control participants, TSS participants have a more adverse macrovascular profile [eg, increased carotid and aortic intima-media thickness (IMT), increased pulse wave velocity (PWV) and decreased carotid artery distensibility and compliance] and retinal microvascular profile (eg, wider venules, narrower arterioles, reduced fractal dimension and increased tortuosity).

PATIENTS AND METHODS

We performed a case-control study including participants aged 6–30 years who had TSS at least 2 years previously and control participants of similar age and sex, recruited from The Royal Children's Hospital Melbourne and Monash Medical Centre, Melbourne, Australia. Cases fulfilled the Centers for Disease Control and Prevention case definition for either probable or definite TSS.[6] Exclusion criteria were pregnancy, diabetes, known atherosclerotic cardiovascular disease, treatment for hypertension and/or hyperlipidemia and chronic auto-immune inflammatory conditions. The study was approved by the human research ethics committee of both hospitals, and written informed consent was obtained from the parents or adult participants.

All data were collected at a single visit after a minimum of 6 hours of fasting. Demographic data and anthropometric measurements (BC 418, Tanita, Tokyo, Japan) were obtained. Pubertal status was based on self-reported Tanner stage. The mean of 3 blood pressure measurements (SphygmoCor XCEL, AtCor Medical, NSW, Australia) was recorded. Blood was collected for measurement of high-sensitivity C-reactive protein (Abbott Architect, IL), glucose, triglycerides, total cholesterol, high density lipoprotein and low density lipoprotein cholesterol (Vitros 5600, Ortho-Clinical Diagnostics, NJ) during the study visit.

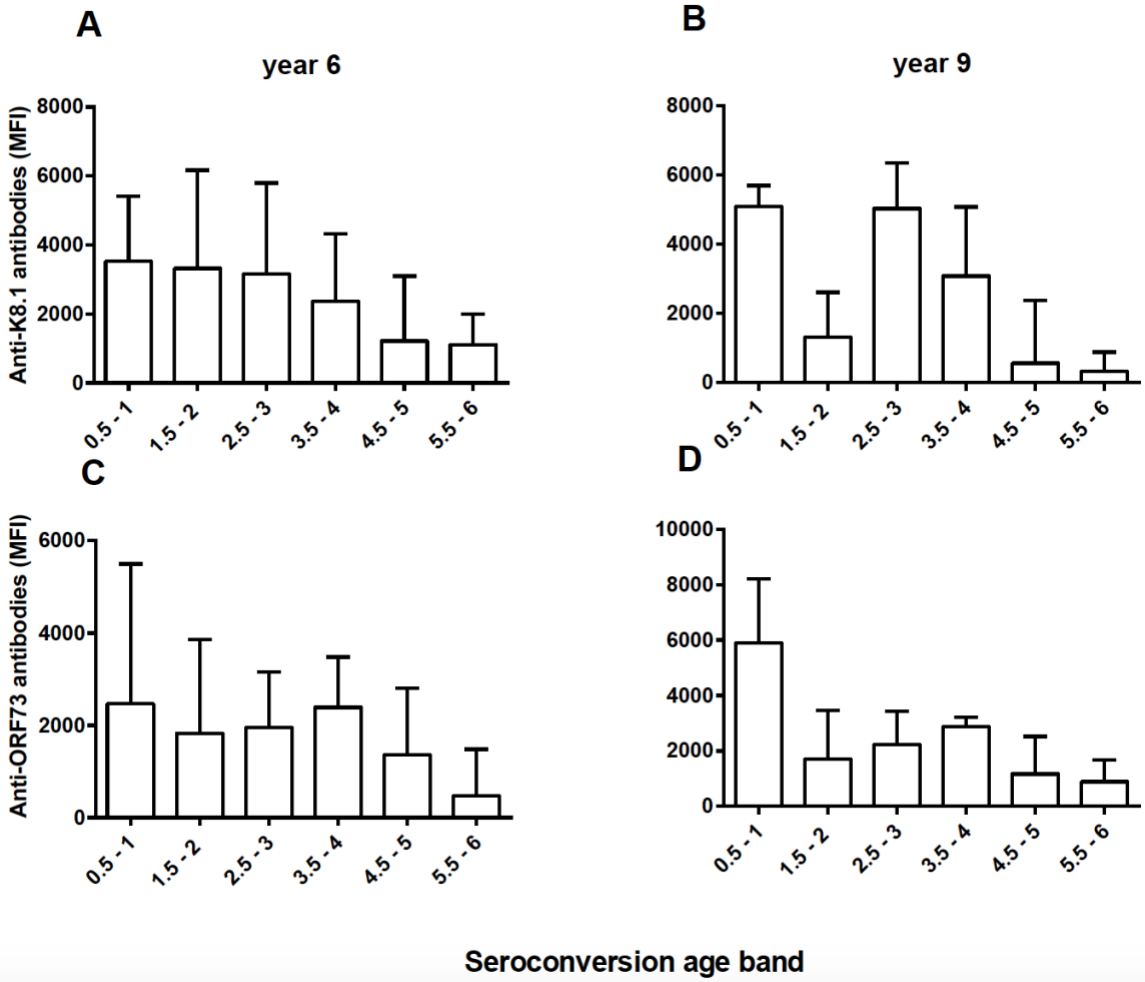
Carotid and Aortic Intima-Media Thickness

Ultrasound images of the carotid artery and the abdominal aorta were acquired using Vivid i (General Electronics Healthcare, Little Chalfont, UK) with simultaneous electrocardiogram (ECG) gating as previously described.[7,8] Cine loops of at least 5 cardiac cycles focused on the intima-media complex of the posterior wall of the right common carotid artery 1 cm proximal to the carotid bulb were recorded for offline analysis. Imaging was focused on the distal 10–15 mm of the abdominal aorta.

The IMT of the far wall 1 cm from the carotid bulb was measured at end diastole using a semiautomated software, Carotid Analyzer for Research (Medical imaging applications LLC, Coralville, IA). The "mean IMT" refers to the average IMT in the selected area of measurement while the "maximum IMT" to the thickest IMT measurement within that segment.[8] The mean of these "mean IMT" or "maximum IMT" measurements from 5 end-diastolic frames was used in analyses.[8]

The 5 best-quality frames of the abdominal aorta were selected from the recorded cine loops for analysis using the same procedure. All IMT measurements were performed by a single grader blinded to subject status. The intrarater reliability was assessed on 10 masked subjects. Intraclass correlation for carotid IMT and aortic IMT was 0.92 [95% confidence interval (CI): 0.69–0.98] and 0.89 (95% CI: 0.59–0.97), respectively.

Chapter Three supplementary material



Supplementary Figure 1: The medians and interquartile ranges of both anti-K8.1 and anti-ORF73 antibodies at ages 6 and 9 for each seroconversion age group/band. Figure 1A and B anti-K8.1 antibodies at ages 6 and 9 respectively, Figure 1C and D anti-ORF73 antibodies at ages 6 and 9 respectively, MFI Median Fluorescence Intensity

Chapter Three - additional results: Age of infection and antibody levels to K10.5, ORF19, ORF61, ORF38, K5, and ORF65 proteins

At age six years, a younger seroconversion age was associated with higher antibody levels to ORF65 (Table 1). Antibodies to other antigens were not significantly associated with the age of seroconversion at age six years (Table 1). Using random effects longitudinal modelling (which determines the association between age of infection and antibody levels that is independent of duration of infection), younger seroconversion age was associated with antibody levels to all six KSHV antigens at all the six-time points (1, 2, 3, 4, 5, and 6) combined (Table 2). Similar to K8.1 and ORF73, other KSHV antigens may elicit antibody levels whose magnitude may be affected by the age at primary infection.

Table 1: Association between the age of KSHV seroconversion, and antibody levels at age six years

	Crude		adjusted[c]	
	GMR[a] (95% CI[b])	P value	GMR[a] (95% CI[b])	P value
K10.5 Seroconversion	0.96 (0.91, 1.08)	0.843	0.99 (0.91, 1.08)	0.829
ORF19 Seroconversion	1.04 (0.98, 1.10)	0.243	1.04 (0.97, 1.10)	0.266
ORF61 Seroconversion	1.00 (0.94, 1.06)	0.948	1.00 (0.94, 1.06)	0.917
ORF38 Seroconversion	1.03 (0.95, 1.11)	0.502	1.02 (0.95, 1.10)	0.548
K5 Seroconversion	1.01 (0.95, 1.07)	0.746	1.01 (0.95, 1.07)	0.773
ORF65 Seroconversion	0.87 (0.82, 0.93)	<0.0001	0.88 (0.82, 0.93)	<0.0001

[a]: Geometric Mean Ratios, [b]: Confidence Intervals, [c]: adjusted for sex. linear regression modelling was used for statistical analysis.

Table 2: Association between age of KSHV seroconversion, age and antibody levels at age one to six years

	Crude		adjusted[c]	
	GMR[a] (95% CI[b])	P value	GMR (95% CI)	P value
K10.5				
Seroconversion	0.92 (0.86, 0.99)	0.022	0.86 (0.80, 0.93)	<0.0001
age	1.12 (1.08, 1.16)	<0.0001	1.22 (1.17, 1.3)	<0.0001
ORF19				
Seroconversion	0.94 (0.90, 0.99)	0.016	0.89 (0.85, 0.94)	<0.0001
age	1.11 (1.08, 1.14)	<0.0001	1.18 (1.14, 1.2)	<0.0001
ORF61				
Seroconversion	0.91 (0.87, 0.95)	<0.0001	0.89 (0.85, 0.93)	<0.0001
age	1.01 (0.98, 1.03)	0.611	1.08 (1.05, 1.11)	<0.0001
ORF38				
Seroconversion	0.90 (0.85, 0.95)	<0.0001	0.88 (0.83, 0.94)	<0.0001
age	0.97 (0.94, 1.00)	0.041	1.05 (1.19, 1.09)	0.003
K5				
Seroconversion	0.87 (0.82, 0.91)	<0.0001	0.86 (0.86, 0.91)	<0.0001
age	0.91 (0.88, 0.94)	<0.0001	1.01 (0.98, 1.05)	0.417
ORF65				
Seroconversion	0.83 (0.80, 0.86)	<0.0001	0.83 (0.80, 0.86)	<0.0001
age	0.95 (0.93, 0.97)	<0.0001	1.01 (0.99, 1.04)	0.229

[a]: Geometric Mean Ratios, [b]: Confidence Intervals, [c]: adjusted for sex and age in years. Random effects modelling was used for statistical analysis.

Chapter Four: Relationship between anaemia, malaria coinfections and KSHV seropositivity in a population-based study in rural Uganda

Preamble

Work published from our group has shown that malaria and hookworm infections were associated with KSHV seropositivity in an urban setting in Uganda. Both malaria and hookworm cause anaemia as well as other complications. Additionally, hypoxia, which may be caused by anaemia, has been shown to reactivate KSHV *in vitro*. Furthermore, we had observed very high seropositivity of KSHV in rural Uganda. We, therefore, investigated the association between haemoglobin levels/anaemia as well as malaria with KSHV seropositivity and antibody levels in rural Uganda, where KSHV is highly prevalent.

These results show that both malaria and anaemia independently increase the risk of being KSHV seropositive. The KSHV association with malaria is larger and stronger compared to that of anaemia. Malaria also partially confounded the association of anaemia with KSHV seropositivity, but anaemia did not confound the association of malaria with KSHV seropositivity. This implies that both factors may play a role in KSHV infection. The work was published in the Journal of Infectious Diseases.

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SECTION A – Student Details

Student	Angela Nalwoga
Principal Supervisor	Stephen Cose
Thesis Title	Determinants of Kaposi's associated sarcoma herpesvirus seropositivity, viral DNA detection and cellular immune responses in Uganda

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	The Journal of Infectious Diseases		
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The Journal of Infectious Diseases

BRIEF REPORT

Relationship Between Anemia, Malaria Coinfection, and Kaposi Sarcoma-Associated Herpesvirus Seropositivity in a Population-Based Study in Rural Uganda

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We examined anemia and malaria as risk factors for Kaposi sarcoma-associated herpesvirus (KSHV) seropositivity and antibody levels in a long-standing rural Ugandan cohort, in which KSHV is prevalent. Samples from 4134 children, aged 1–17 years, with a sex ratio of 1:1, and 3149 adults aged 18–103 years, 41% of whom were males, were analyzed. Among children, malaria infection was associated with higher KSHV prevalence (61% vs 41% prevalence among malaria infected and uninfected, respectively); malaria was not assessed in adults. Additionally, lower hemoglobin level was associated with an increased prevalence of KSHV seropositivity, both in children and in adults.

Keywords. Kaposi sarcoma herpesvirus antibodies; rural population; anemia; malaria.

We recently reported Kaposi sarcoma-associated herpesvirus (KSHV) prevalence of 95% among adults in a rural population cohort (the General Population Cohort [GPC] in Uganda [1]), the highest prevalence of KSHV ever reported, in addition to high KSHV antibody levels. We propose that the very high prevalence in this population may be driven by frequent KSHV reactivation, viral shedding, and transmission rates and that the high antibody levels also reflect frequent reactivation. It is important, therefore, to study potential cofactors for reactivation in relation to KSHV prevalence and antibody levels. We have previously reported an association between malaria and hookworm infections and KSHV seropositivity in an urban population in Uganda [2, 3]. Because both malaria and hookworm are associated with anemia, we hypothesized that anemia may have a role in KSHV transmission via viral reactivation. This hypothesis is supported by data from in vitro experiments showing reactivation of KSHV in conditions of hypoxia [4]. In this study we aimed to confirm the high prevalence of KSHV in the GPC in recent years, with higher antiretroviral therapy coverage, and determine the role of anemia and malaria coinfection as risk factors for KSHV prevalence and antibody levels in a highly endemic population.

METHODS

Study Population and Sociodemographic Data Collection

The GPC is located in south-western Uganda, in Kyamulibwa subcommunity of Kalungu district, with an altitude of approximately 1200 m above sea level. It is community-based open cohort of about 22 000 people in 25 adjacent villages [5]. This cross-sectional study analyzed plasma samples collected from 2 surveys, the adult survey and the children survey. The adults were surveyed in 2014/2015 and the children in 2016. Adults without hematological parameter data and children without either hematological parameter data or malaria parasitemia status data were excluded in the laboratory analysis. Children less than 1 year of age were excluded from the statistical analysis and children less than 2 years were not tested for HIV serostatus, due to the potential for maternal IgG to be present, which could affect antibody measurement. Socioeconomic scores were generated for adults using principal component analysis of various household indicators during the previous survey.

Ethical Approval

The study was approved by the Research and Ethics Committee Uganda Virus Research Institute and the Uganda National Council for Science and Technology.

Hematological and Serological Analysis

During these 2 surveys, blood was collected from study participants and tested immediately after collection for human immunodeficiency virus (HIV); a smaller proportion of samples were also tested for malaria parasitemia and hemoglobin levels, using point-of-care assays and rapid tests. HIV serostatus was determined using rapid diagnostic tests. Malaria parasitemia was measured in children only, using malaria Rapid Diagnostic Tests (One Step Malaria HRP-II [Pf] and pLDH [Pan] Antigen Rapid Test). Hemoglobin levels in g/dL were obtained from a Hemocue 201 analyzer.

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Stored plasma samples for both children and adults were retrieved and tested for KSHV antibodies using an in-house enzyme-linked immunosorbent assay (ELISA), as previously described [6]. Samples from the 2 surveys were tested separately after simple randomization onto ELISA plates. Antibodies to both K8.1 and ORF73 proteins were measured as optical density. Each ELISA plate contained 3 negative and positive control wells; negative controls were used to calculate a cutoff value for every plate as previously described [2, 7]. Seropositivity was defined as reactivity to either K8.1 or ORF73 proteins.

Statistical Analysis

Statistical analysis was carried out using STATA13 (Statacorp, College Station, TX). Children's and adults' results were analyzed separately. Hemoglobin levels were mean centered for easier interpretation. Anemia was defined using hemoglobin levels in g/dL after altitude adjustment following World Health Organization (WHO) guidelines [8]. A constant value 0.5 was subtracted from hemoglobin levels for altitude adjustment, the results were then categorized into normal and anemic using the following cutoff values: 11.0 for pregnant females and children below 5 years, 11.5 for children 5 to 11 years, 12.0 for children 12 to 14 years and other females 15 years and above, and 13.0 for males 15 years and above. These WHO hemoglobin reference ranges used to define anemia may not be representative of African populations, as previously reported [9, 10], because they are based on western population data. We therefore analyzed hemoglobin both as a continuous variable and as categorized into normal and anemic using separate regression models.

Linear regression with bootstrapped confidence intervals (CI) was used for antibody levels analysis, because they were severely skewed. Logistic regression was used for seroprevalence analysis; furthermore, we adjusted for clustering at the village level using survey commands. We assessed interaction between age and hemoglobin levels, as well as between age and anemia, in relation to anti-K8.1 antibody levels, anti-ORF73 antibody levels, and KSHV prevalence based on a priori suspicions of interaction, using likelihood ratio tests.

RESULTS

The characteristics of the individuals analyzed are shown in Supplementary Table 1. We analyzed results from 3149 adults and 4134 children. This analysis included children aged 1 to 17 years and adults aged 18 to 103 years (Supplementary Table 1).

Risk Factors for KSHV Prevalence and Antibody Levels Among Adults

KSHV prevalence was 91% in all adults (2871/3149) (Supplementary Figure 1). Every 1 g/dL decrease in hemoglobin values was associated with increased odds of being KSHV seropositive (odds ratio [OR] = 0.86; 95% CI, 0.77–0.96; P = .006) and anemic individuals were more likely to be KSHV seropositive compared to people with normal hemoglobin values, but this association was not statistically significant (OR = 1.25; 95% CI, 0.87–1.79; P = .229; Table 1).

We then analyzed antibody levels to K8.1 and ORF73 proteins as continuous variables without categorizing participants as seropositive or seronegative. Anemic adults had higher antibodies to ORF73 protein compared to individuals with normal hemoglobin values (regression coefficient 0.28; 95% CI, 0.16–0.39; P < .0001). Similarly, every 1 g/dL decrease in hemoglobin was associated with an increase in ORF73 antibody optical densities (Table 1). The association between hemoglobin and antibodies to ORF73 protein was strongest among older people (Table 1). Conversely, anti-K8.1 antibody levels were not significantly associated with either hemoglobin levels or anemia (Table 1). This may be due to the relative abundance of LANA compared to late lytic proteins such as K8.1, even during KS disease [11].

Compared to HIV-negative adults, HIV-positive adults had lower antibodies to KSHV, especially those with CD4 counts of 500 cell/μL or less (Table 1). This may be due to B-cell dysfunction caused by HIV infection, and consequent decreased antibody responses [12, 13]. On the other hand, HIV-positive adults with CD4 counts above 500 cells/μL, compared to HIV-negative adults, were more likely to be KSHV seropositive (Table 1), which may be due to antiretroviral treatment.

Risk Factors for KSHV Prevalence and Antibody Levels Among Children

We then investigated associations between KSHV prevalence and antibody levels and risk factors among children. Overall, KSHV prevalence was 51% (2117/4134) in the children, the prevalence increased with age, rising from 31% among 1–5 year olds, to 53% among 6–12 year olds, to 73% among 13–17 year olds (Supplementary Figure 1). We first adjusted for HIV status, age, and sex, then malaria parasitemia and anemia/hemoglobin levels were added in the full models. In the first analysis, hemoglobin levels, malaria parasitemia, and age were strongly associated with KSHV prevalence (Table 2). Every 1 g/dL decrease in hemoglobin levels increased the odds of being KSHV seropositive by 11% (P < .0001) and the odds of being KSHV positive if anemic compared to normal hemoglobin levels was 1.42 (95% CI, 1.18–1.71; P < .0001). The odds of being KSHV seropositive, if malaria infected, compared to uninfected was 2.22 (95% CI, 1.84–2.69; P < .0001) and every annual increase in age was associated with a 17% increased risk of being KSHV seropositive (Table 2).

After adjusting for malaria parasitemia, the risk of being KSHV seropositive for every 1 g/dL decrease in hemoglobin reduced to 7% (P = .005). Similarly, the odds of being KSHV seropositive in comparing anemic children to children with normal hemoglobin levels reduced to 1.23 (95% CI, 1.01–1.49; P = .037). After adjusting for hemoglobin levels, the odds of being KSHV seropositive comparing children with and

Table 1. Risk Factors for KSHV Prevalence and Antibody Levels Among Adults Aged 18 to 103 Years

Risk Factors for KSHV Prevalence

Risk Factor	KSHV Prevalence	Adjusted[a] OR (95% CI)	P Value
Age		1.01 (1.003–1.02)	.010
Sex			
Males	94% (1218/1297)	1	
Females	89% (1653/1852)	0.61 (0.46–0.80)	.001
HIV and CD4 count status			
(–) CD4 count unknown	92% (2615/2841)	1	
(+) CD4 count > 500 cells/μL	70% (32/46)	0.16 (0.10–0.26)	<.0001
(+) CD4 count ≤ 500 cells/μL	90% (76/84)	0.61 (0.16–2.36)	.451
(+) CD4 count unknown	83% (146/175)	0.53 (0.29–0.96)	.039
Hemoglobin levels[b]		0.86 (0.77–0.96)	.006
Anemia[c]			
Normal	91% (2153/2370)	1	
Anemic	92% (718/779)	1.25 (0.87–1.79)	.229

Risk Factors for Anti-K8.1 and Anti-ORF73 Antibody Levels

Risk Factor	K8.1		ORF73	
	Adjusteda Coef. (95% CI)	P Value	Adjusteda Coef. (95% CI)	P Value
Age	0.004 (0.001–0.007)	.015	0.01 (0.007–0.013)	<.0001
Sex				
Males	Ref			
Females	−0.25 (−0.35 to −0.14)	<.0001	−0.24 (−0.34 to −0.14)	<.0001
HIV and CD4 count status				
(–) CD4 count unknown	Ref		Ref	
(+) CD4 count > 500 cells/μL	−0.77 (−1.21 to −0.33)	.001	−0.71 (−1.15 to −0.29)	.001
(+) CD4 count ≤ 500 cells/μL	−0.33 (−0.64 to −0.02)	.036	−0.48 (−0.79 to −0.17)	.003
(+) CD4 count unknown	−0.28 (−0.51 to −0.06)	.014	−0.38 (−0.59 to −0.16)	.001
Hemoglobin levels[b]	−0.03 (−0.06 to 0.01)	.119		
Anemia[c]				
Normal	Ref			
Anemic	0.007 (−0.11 to 0.13)	.910	0.28 (0.16–0.39)	<.0001

Age Group Specific Association Between Anti-ORF73 Antibody Levels and Hemoglobin Levels

	Age Group, years	Adjusteda Coef. (95% CI)	P Value	Interaction P Value
Hemoglobin levels[b]	18–24	−0.09 (−0.16 to 0.02)	.009	
	25–44	−0.08 (−0.12 to 0.03)	.001	
	45–103	−0.17 (−0.21 to 0.12)	<.0001	.007

Anti-K8.1 and anti-ORF73 antibody levels (measured as optical density) were obtained from enzyme-linked immunosorbent assay. KSHV seropositivity was defined as reactivity to either K8.1 or ORF73 antigens. Logistic regression, allowing for the survey design, was used for statistical analysis of risk factors for KSHV prevalence. Linear regression with bootstrapped confidence intervals was used for statistical analysis of risk factors for antibody levels. Age group specific associations between ORF73 antibody levels and hemoglobin levels were reported due to interaction between age and hemoglobin levels.

Abbreviations: Coef., regression coefficient; CI, confidence interval; KSHV, Kaposi sarcoma-associated herpesvirus; OR, odds ratio.

[a]Adjusted for age, sex, HIV status, and household socioeconomic status.

[b]Hemoglobin levels were mean (13.7 g/dL) centered.

[c]Hemoglobin levels were altitude adjusted and categorized into normal and anemic following World Health Organization guidelines.

without malaria parasitemia changed little (OR = 2.13; 95% CI, 1.75–2.58; $P < .0001$) (Table 2). Every annual increase in age remained strongly associated with increased KSHV prevalence risk, OR = 1.18 ($P < .0001$) even after adjusting for malaria parasitemia and hemoglobin (Table 2).

We then finally investigated associations between the same risk factors and KSHV antibody levels (optical density) as continuous variables without categorizing participants as seropositive or seronegative. Only malaria parasitemia was associated with both anti-K8.1 and anti-ORF73 antibody levels in the fully multivariate analysis (Table 2).

Discussion

We observed a significant association between hemoglobin levels and KSHV prevalence among children and adults, where people with low levels of hemoglobin were more likely to be

Table 2. Risk Factors for KSHV Prevalence and Antibody Levels Among Children Aged 1 to 17 years

Risk Factors for KSHV Prevalence					
		Adjusted[a]		Adjusted[b]	
Risk Factor (n)	KSHV Prevalence	OR (95% CI)	P Value	OR (95% CI)	P Value
Hemoglobin levels (3199)[c]		0.89 (0.84–0.93)	<.0001	0.93 (0.88–0.98)	.005
Anemia[d]					
Normal	44% (1143/2584)	1			
Anemic	48% (294/615)	1.42 (1.18–1.71)	<.0001	1.23 (1.01–1.49)	.037
Malaria					
Negative	41% (1088/2630)	1		1	
Positive	61% (348/569)	2.22 (1.84–2.69)	<.0001	2.13 (1.75–2.58)	<.0001
Age (4134)		1.17 (1.15–1.18)	<.0001	1.18 (1.15–1.21)	<.0001
Sex					
Boys	52% (1076/2061)	1		1	
Girls	50% (1041/2073)	0.93 (0.82–1.07)	.27	0.94 (0.82–1.07)	.33
HIV					
Negative	53% (2030/3812)	1		1	
Positive	40% (19/48)	0.49 (0.25–0.96)	.04	0.55 (0.30–1.03)	.06

Risk Factors for Antibody Levels								
	K8.1				ORF73			
	Adjusted[a]		Adjusted[b]		Adjusted[a]		Adjusted[b]	
Risk factor (n)	Coef. (95% CI)	P value	Coef. (95% CI)	P value	Coef. (95% CI)	P value	Coef. (95% CI)	P value
Malaria								
Negative (2630)	ref		ref		ref		ref	
Positive (569)	0.27 (0.18–0.38)	<.0001	0.26 (0.16–0.37)	<.0001	0.30 (0.21–0.38)	<.0001	0.26 (0.18–0.34)	<.0001
Age (4134)	0.08 (0.07–0.09)	<.0001	0.1 (0.08–0.11)	<.0001	0.06 (0.05–0.07)	<.0001	0.05 (0.04–0.06)	<.0001
Hemoglobin levels (3199)[c]	−0.04 (−0.06 to −0.01)	.003	−0.02 (−0.05 to 0.01)	.2	−0.03 (−0.06 to −0.01)	.001	−0.02 (−0.04 to 0.005)	.13
Anemia[d]								
Normal	ref		ref		ref		ref	
Anemic	0.04 (−0.05 to 0.13)	.414	−0.02 (−0.12 to 0.07)	.649	0.12 (0.05–0.19)	.001	0.07 (−0.01 to 0.14)	.073
Sex								
Boys (2061)	ref		ref		ref		ref	
Girls (2073)	−0.05 (−0.11 to 0.02)	.17	−0.01 (−0.09 to 0.06)	.75	0.01 (−0.04 to 0.07)	.63	−0.01 (−0.07 to 0.05)	.66
HIV								
Negative (3808)	ref		ref		ref		ref	
Positive (270)	−0.24 (−0.56 to 0.07)	.13	−0.12 (−0.48 to 0.24)	.5	0.20 (−0.17 to 0.57)	.29	0.29 (−0.13 to 0.72)	.18

Logistic regression, allowing for survey design, was used for statistical analysis of risk factors and KSHV prevalence. Linear regression with bootstrapped confidence interval used in the analysis of risk factors for KSHV antibody levels. ORF73 and K8.1 antibody levels (measured as optical density) were obtained from enzyme-linked immunosorbent assay. KSHV prevalence defined as antibody reactivity to either K8.1 or ORF73 antigens. HIV status obtained using rapid diagnostic tests. Malaria parasitemia determined using rapid diagnostic tests.

Abbreviations: Coef., regression coefficient; CI, confidence interval; KSHV, Kaposi sarcoma-associated herpesvirus; OR, odds ratio.

[a]Adjusted for age, sex, and HIV status.

[b]Adjusted for age, sex, HIV status, hemoglobin, and malaria parasitemia.

[c]Hemoglobin levels were mean (13.0 g/dL) centered.

[d]Hemoglobin levels were altitude adjusted and categorized into normal and anemic following World Health Organization guidelines.

KSHV seropositive. As a categorical variable, anemia was associated with KSHV prevalence among children. Reduction in hemoglobin has been shown to cause hypoxia/low tissue oxygen, while hypoxia has been shown to reactivate KSHV in vitro [4]. We therefore hypothesize that a low hemoglobin level leads to reactivation of KSHV through hypoxia. Increased reactivation may help spread the virus during initial infection. Alternately, hypoxia may enhance initial infection of cells, possibly through upregulation of the replication and transcription activator [14]. In this cross-sectional study, we did not directly measure KSHV reactivation or KSHV viral load in blood or plasma, although antibody levels may be viewed as a surrogate marker for frequent reactivation. The connection between KSHV reactivation, hypoxia, and anemia requires further investigation.

We showed that children infected with malaria are more likely to be KSHV seropositive. Additionally, the effect of anemia and/

or hemoglobin levels on KSHV prevalence and antibody levels reduced to about 50% after adjusting for malaria infection. Malaria causes anemia, and in part the anemia effect in children could be explained (confounded) by malaria infection. The consistent association between malaria infection and KSHV prevalence suggests malaria may be driving KSHV transmission in malaria endemic areas. This might imply that exposure to malaria significantly impacts on KSHV reactivation, which might also have long-lasting effects. The mechanism through which malaria may reactivate KSHV requires further investigation.

Findings from this study suggest malaria infection as a risk factor for KSHV prevalence. Malaria-associated anemia is one mechanism that likely contributes to this association but cannot entirely explain it. In KSHV and malaria endemic areas, a number of other parasite coinfections such as helminths, which cause anemia and/or immunomodulation are common. The role of multiple parasitic infections and KSHV transmission and pathogenesis warrants further careful study.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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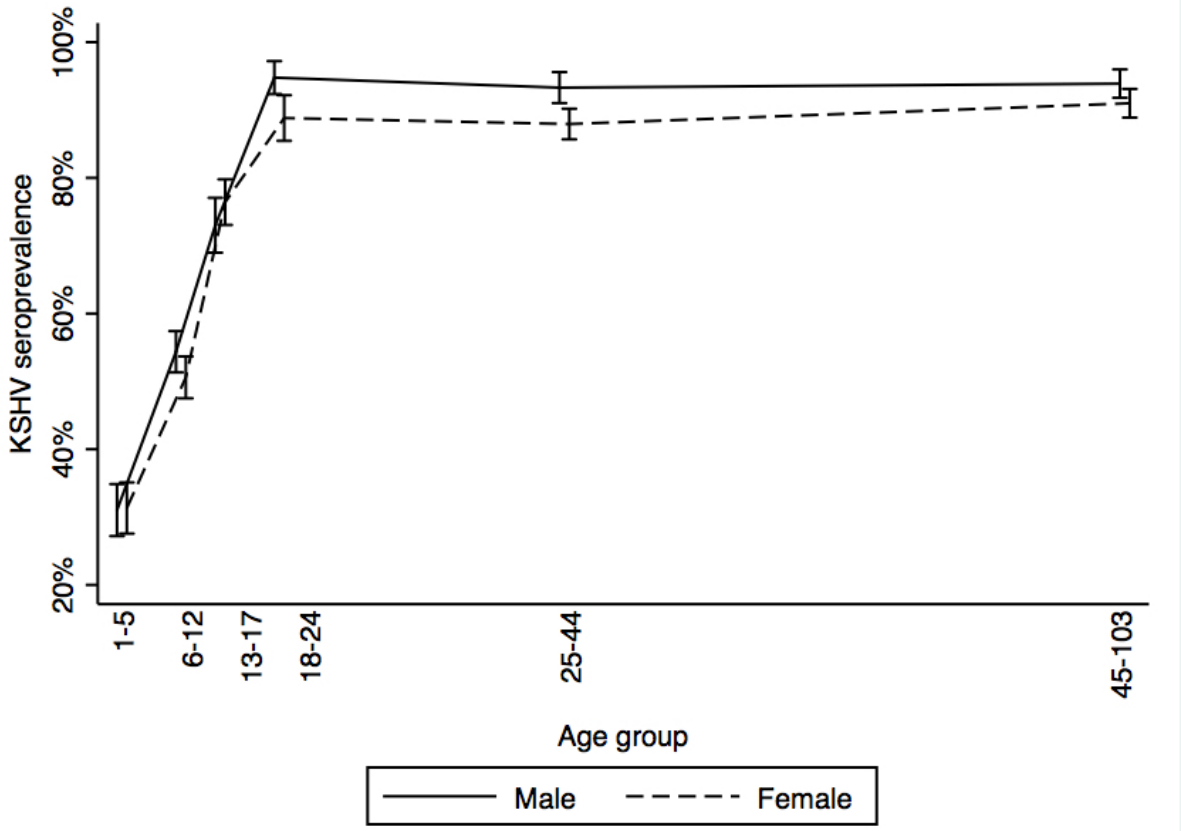
Chapter Four supplementary material



Supplementary Figure 1: KSHV prevalence and 95% confidence intervals across ages 1 to 103 years among individuals from the General Population Cohort in south-western Uganda. KSHV Seropositivity defined as antibody reactivity to either K8.1 or ORF73 antigens. Anti-K8.1 and anti-ORF antibodies detected using ELISA

Supplementary Table 1: General Population Cohort characteristics from the calendar years 2014 to 2016

Children (25th survey, carried out in 2016)	
Age groups (years)	
1 – 5	28% (1148/4134)
6 – 12	49% (2040/4134)
13 – 17	23% (946/4134)
Sex	
Males	50% (2061/4134)
Haemoglobin levels, mean (range)	13.0 (3.7, 21.8) g/dL
Anaemia[a]	
Normal	81% (2584/3199)
Anaemic	19% (615/3199)
HIV prevalence	1% (48/3860)
Malaria parasitaemia	18% (569/3199)
Adults (24th survey, carried out in 2014 and 2015)	
Age groups	
18 – 24	21% (666/3149)
24 - 44	40% (1265/3149)
45 - 103	39% (1218/3149)
Sex	
Males	41% (1297/3149)
Haemoglobin levels, mean (range)	13.7 (2.8, 21.1) g/dL
Anaemia[a]	
Normal	75% (2370/3149)
Anaemic	25% (779/3149)
HIV prevalence	9.7% (305/3146)

Proportions were determined using survey commands. [a]haemoglobin levels were altitude adjusted and categorised into normal and anaemic following 2011 WHO guidelines.

Chapter Four - additional results: KSHV seropositivity in children including those aged below one year

All the seropositivity and antibody data shown in this thesis excluded children below one year of age. This was because in those below one-year maternal IgG antibodies were likely to be present, confounding the data (Figure 1).

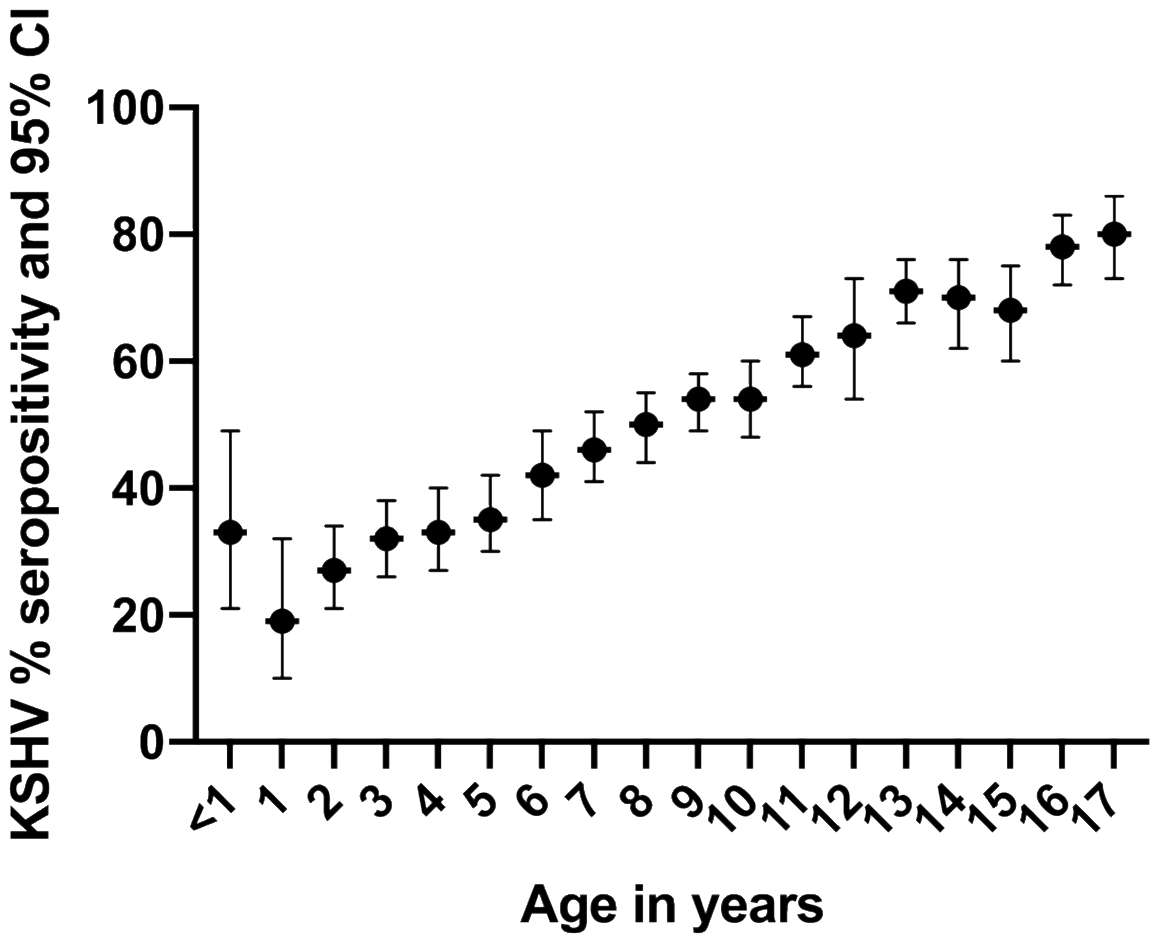


Figure 1: KSHV seropositivity and 95% confidence intervals among individuals from the General Population Cohort (GPC) including those below one year of age. Seropositivity determined from IgG antibody to K8.1 and ORF73 measurement using ELISA. Data analysis was done in STATA adjusting for survey design.

Chapter Five: Kaposi's sarcoma-associated herpesvirus prevalence and parasite infections in Ugandan fishing communities on Lake Victoria islands

Preamble

KSHV prevalence varies even within small geographical areas. Lake Victoria fishing villages have a high prevalence of both HIV and *Schistosoma mansoni* infection. The prevalence of KSHV and its associated risk factors in these areas have not been determined before. Previously, *in vitro* studies showed reactivation of KSHV by *S. mansoni*. We aimed to determine the prevalence of KSHV in the Lake Victoria island communities of Uganda. We also aimed to determine the effect of *S. mansoni* and other parasitic infections, including malaria, on KSHV seropositivity and antibody levels. Using a well-established community-based cohort we planned to ascertain the effect of intensive vs. standard anti-helminthic treatment on KSHV antibody levels.

S. masoni infection was associated with KSHV seropositivity at both baseline and after three years of anti-helminthic treatment. Among other parasitic infections, malaria was associated with KSHV seropositivity after three years of anti-helminthic treatment. We did not observe any effect of intensive vs. standard anti-helminthic treatment on KSHV antibody levels. These data have been submitted to PLOS Neglected Tropical Diseases.

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Student	Angela Nalwoga
Principal Supervisor	Stephen Cose
Thesis Title	Determinants of Kaposi's sarcoma associated herpesvirus seropositivity, viral DNA detection and cellular immune responses in Uganda

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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Where was the work published?			
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Where is the work intended to be published?	Plos Neglected Tropical Diseases
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Stage of publication	**Submitted**

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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	

Student Signature: [redacted] **Date:** 20th May 2019

Supervisor Signature: [redacted] Date: 21/05/2019

Kaposi's sarcoma-associated herpesvirus prevalence is associated with helminth infections in Ugandan fishing communities on Lake Victoria islands

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Abstract

We investigated the impact of helminths and malaria infection on Kaposi's sarcoma associated herpesvirus (KSHV) seroprevalence, using samples and data collected from a cluster-randomised trial of intensive versus standard anthelminthic treatment. The trial was carried out among fishing communities on Lake Victoria islands in Uganda. Plasma samples from 1571 participants in a household survey, collected after three years of the trial intervention, were tested for KSHV IgG antibody responses using ELISA. Infection by helminths (*S. mansoni*, *N. americanus*, *T. trichiura* and *S. stercoralis*) was diagnosed using real time PCR, urine circulating cathodic antigen (CCA) and stool microscopy (Kato-Katz method) while malaria infection was diagnosed using microscopy. We analysed the relationship between helminth and malaria infections and KSHV seropositivity using regression modelling, allowing for survey design.

Around half of the participants were male (48%) and the median age was 24 years. The most prevalent helminth infection was *S. mansoni* (34% by microscopy 86% by CCA and 50% by PCR). KSHV seropositivity was 56% among those 1-12 years and >80% in those 13+ years; malaria parasitaemia prevalence was 4%. Among the infections tested, *S. mansoni* (by microscopy, adjusted Odds Ratio (aOR=1.43 (1.04 -1.95), p=0.028) and malaria parasitaemia (aOR=3.49 (1.08 – 11.28), p=0.038) were positively associated with KSHV seroprevalence. Additionally, KSHV seropositive participants had higher *S. mansoni*-specific IgE and IgG antibody concentrations in plasma.

Schistosoma species skew the immune response towards Th2 and regulatory responses, which could impact on an individual's susceptibility to KSHV and its reactivation if co-infected with both organisms.

Keywords: Kaposi's sarcoma associated herpesvirus (KSHV), *Schistosoma mansoni*, malaria parasitaemia, Lake Victoria islands, Uganda

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Author summary

Kaposi's sarcoma associated herpesvirus (KSHV) the causative agent of Kaposi's sarcoma cancer varies geographically. KSHV infections tend to spread the highest in sub-Saharan Africa with Uganda having the highest occurrence of the infection reported. Infection with

KSHV is lifelong with an intermittent revival of the virus leading to viral spread. In this study, we show that infection with *Schistosoma mansoni* and malaria parasites is associated with being infected or exposed to KSHV. These parasite infections interfere with the proper functioning of the immune system to control viral infections. This might lead to the revival of KSHV in infected people or make uninfected children susceptible to infection, hence increasing the spread of KSHV in sub Saharan Africa.

Introduction

The prevalence of Kaposi's sarcoma associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8), varies geographically, unlike that of other herpesviruses which are ubiquitous [1-3]. Uganda has a high prevalence of KSHV [4, 5] and a high incidence of Kaposi's sarcoma (KS) [6, 7]. The incidence of KS rises dramatically among immunocompromised individuals [8-10]; immunosuppression has been implicated in the reactivation of KSHV and the progression of KS [9, 11].

Co-infection with helminths has been shown to modulate immune responses to other infections and vaccines [12-14]. Chronic infection with *Schistosoma* is characterised by the production of IL4, IL5 and IL13 cytokines, typical of a T helper (Th) type 2 response and IL10, a regulatory cytokine [15, 16]. The skewed immune response to a Th2 and regulatory response may impair the T helper (Th) 1 response, vital for control of viral infections [17-19]. The impact of *Schistosoma* co-infection on herpesviruses and other viruses has been demonstrated in animal models where *Schistosoma mansoni* infection led to IL4-mediated reactivation of murine herpesvirus 68 and M2 macrophage polarization [17, 18].

Our group has documented associations between KSHV antibodies and parasite infections including *P. falciparum* and helminths (hookworm and *Mansonella perstans*) in rural [4] and peri-urban [20-22] populations in Uganda. Lake Victoria island communities in East Africa are characterised by poor sanitation and a high prevalence of infectious diseases, including

schistosomiasis [23-26]. No study to date has documented the burden of KSHV or KS in these unique communities. Therefore, this study aimed to determine the seropositivity of KSHV in the Lake Victoria island communities of Koome sub-county, Uganda and the association between KSHV seropositivity and parasite co-infections.

Methods

Study population, participant selection and ethical approval

This cross-sectional study used samples from an open cluster-randomised trial of intensive versus standard anthelminthic treatment, the Lake Victoria Island Intervention Study on Worms and Allergy-related diseases (LaVIISWA) (ISRCTN47196031) [29]. The study was carried out in the Lake Victoria island communities of Koome sub-county, Mukono district, Uganda. Twenty-six island villages were included in the trial. These 26 villages were randomised to receive either standard or intensive anthelminthic treatment, with 13 villages in each trial arm [29, 30]. After three years of the intervention, a household survey was conducted, which involved selecting a random sample of 70 households per village and inviting all residents of selected households to participate. Data on socio-demographic characteristics were collected, clinical examinations were performed, and blood and stool samples were taken. A total of 1571 plasma samples from the survey were selected randomly for this analysis. Ethical approvals were obtained from the Uganda Virus Research Institute Research and Ethics Committee (reference number: GC/127/17/04/317), the Uganda National Council for Science and Technology (reference number: HS1183) and the London School of Hygiene & Tropical Medicine Research and Ethics committee (reference number: 9917-9). Written informed consent was obtained from all adults aged 18 years and above. Children below 18 years were consented into the study by their parents or guardians; we also sought, in addition to parental consent, written assent from children aged between 8-17 years.

Serology

IgG antibodies to K8.1 and ORF73 antigens were measured using ELISA to determine KSHV seroprevalence, as previously described [30]. Each plate contained three negative and positive controls. The negative controls were used to determine the cut-off value. Seropositivity was defined as reactivity to either K8.1 or ORF73 antigens or both. IgE, IgG (all subclasses) and IgG4 antibody concentrations to *Schistosoma mansoni* Egg Antigen (SEA) and *Schistosoma mansoni* adult Worm Antigen (SWA) were measured using ELISA. Antigen concentrations of 8 μg/mL (SWA) and 2.4 μg/mL (SEA) plus sample dilutions of 1/20 (IgE), 1/200 (IgG4) and 1/3000 (IgG) were used, as previously reported [14, 31, 32].

Diagnostic testing and socio demographic data collection

Duplicate slides were made from each stool sample and analysed independently by two technicians for helminth infection and intensity using the Kato-Katz (KK) method [33]. An aliquot of the stool was stored in 70% ethanol and later used to detect helminths by real time PCR. The multiplex real time PCR assay was used to detect *Necator americanus*, *Strongyloides stercoralis* and *Schistosoma mansoni* as previously described [34, 35]. *S. mansoni* antigens were also tested in urine using the Circulating Cathodic Antigen (CCA) kits (Rapid Medical Diagnostics, Pretoria, South Africa) [29]. *Mansonella perstans* infection was detected using the modified Knott's method [36] and thick blood films were made for malaria parasitaemia detection using microscopy. Questionnaires were administered for demographic data collection while HIV infection status was determined using rapid HIV diagnostic kits following the Uganda national HIV testing algorithm.

Statistical analysis

Statistical analysis was carried out using STATA version 13 (Statacorp, College Station, Texas USA). Logistic regression (allowing for the survey design) was used to determine associations between risk factors and KSHV seroprevalence. A p value of less than 5% was

considered statistically significant. Multivariable models included age (grouped), sex, HIV status, *S. mansoni*, hookworm and malaria parasite infection. Participants not tested for HIV were also included in the analysis. Observational analyses took into account clustering within villages and village-level weights to allow for the different village sizes. [37]. For assessing the effect of intensive versus standard treatment, the analysis was done at the cluster level. The proportion of KSHV seropositive participants was calculated for each village, and the mean of these taken for the two trial arms. The risk ratio (RR) was then calculated by dividing the mean KSHV prevalence in the intensive arm by that in the standard arm, and a Taylor approximation was used to calculate a 95% confidence interval for this RR. The p-value was generated from a t-test comparing the village-level prevalences between the two arms. A similar approach was used to assess the effect of intensive versus standard treatment on KSHV antibody levels.

Results

Participants Characteristics

A total of 1571 individuals from 26 villages were analysed. The median age was 24 years with an interquartile range (IQR) of 9 to 33 years. The overall proportion of males was 48%. Details of the socio-demographic characteristics of the study population are shown in Table 1. Around a quarter of participants (342/1571, 26%), mainly children, were not tested for HIV. There were 201 HIV seropositive individuals among 1229 tested (17% prevalence), with 103 participants confirmed to be on antiretroviral therapy (ART). Malaria infection prevalence was low, at 4% overall, and 8% in children aged 1 to 12 years. Among the helminth infections tested, *Schistosoma mansoni* was the most prevalent, as expected, due to the close proximity of the study sites to the waters of Lake Victoria. The prevalence was 86% by CCA, 50% by PCR and 34% by microscopy (Table 1). Hookworm prevalence was 2% by microscopy and 8% by PCR. The prevalence of other helminths was 6% for *Strongyloides stercoralis* (using PCR), 9% for *Trichuris trichiura* (using KK), 0.04% for

Ascaris lumbricoides (using KK) and 0.9% for *Mansonella perstans* (Table 1). The majority of the infected individuals had light to moderate helminth infections; 8% had a heavy *S. mansoni* infection based on KK (Table 1).

Table 1: Characteristics of the study population

Factor	**n=1571**
Age, median and interquartile range	24 (9 – 33)
Age group, years	
1-12	31% (492/1571)
13-30	39% (596/1571)
31-44	22% (353/1571)
45-72	9% (130/1571)
Sex (males)	48% (801/1571)
HIV prevalence	
Overall	17% (201/1229)
Participants aged 1-12 years	2% (6/270)
Participants aged 13-72 years	21% (195/959)
ART status	
(+) treated	57% (103/201)
(+) untreated	6% (13/201)
(+) no treatment status	37% (85/201)
Malaria infection	
Overall	4% (60/1554)
Children (1-12 years)	8% (34/491)
Schistosoma mansoni prevalence	
KK	34% (440/1355)
PCR	50% (673/1353)
CCA	86% (1225/1430)
Schistosoma mansoni intensity KK	
Uninfected	66% (915/1355)
Light infection	16% (216/1355)
Moderate	10% (119/1355)
Heavy	8% (105/1355)
Necator americanus prevalence PCR	8% (120/1353)
Hookworm prevalence KK	2% (30/1355)
Strongyloides stercoralis prevalence PCR	6% (98/1353)
Trichuris trichiura prevalence KK	9% (135/1355)
Ascaris lumbricoides prevalence KK	0.04% (8/1355)
Mansonella perstans prevalence	0.9% (14/1567)

Proportions were weighted to allow for the survey design. Helminths infection status and were determined from a single stool sample using Kato-Katz (KK) method or PCR (polymerase chain reaction) method or both. Rapid tests were used for HIV screening and microscopy was used for malaria diagnosis.

KSHV seropositivity

Overall KSHV seropositivity was 75%. The prevalence increased steeply with age (overall p<0.0001), rising from 56% in the 1-12-year age group to 84% in the 13-30-year age group, and plateaued thereafter (Figure 1).

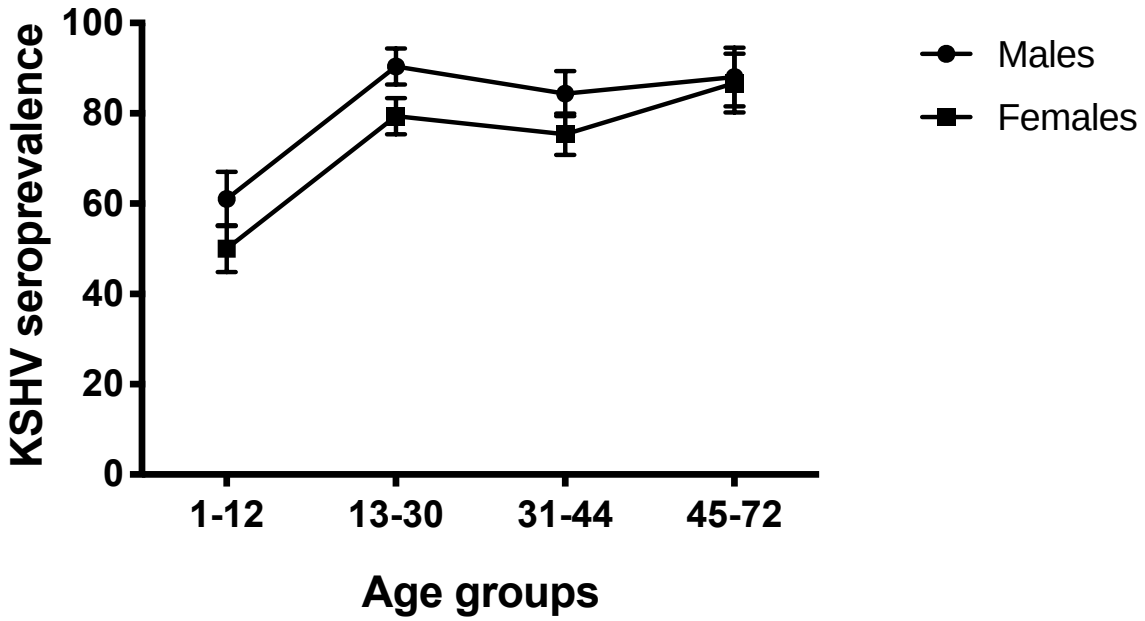

Figure 1: KSHV seropositivity and 95% confidence intervals (CI) across ages 1 to 72 years. KSHV Seropositivity defined as reactivity to either ORF73 or K8.1 proteins. KSHV antibodies were detected using ELISA. Seropositivity and 95% CI were obtained in STATA, allowing for the survey design.

Associations between KSHV seropositivity and risk factors

We investigated associations of KSHV seropositivity with parasite infections and other factors. Overall, KSHV prevalence was higher in males compared to females (adjusted Odds Ratio (aOR)=1.72 (1.29, 2.30), p=0.001) (Table 2). HIV seropositive individuals on ART were less likely to be KSHV seropositive compared to HIV seronegative individuals (Table 2).

Table 2: Association between KSHV seropositivity and risk factors

Factor	KSHV prevalence	Crude OR[a] (95% CI[b])	P value	Adjusted[c] OR (95% CI)	P value
Age group (years)					
1-12	56% (289/492)	1		1	
13-30	84% (511/596)	4.35 (2.80,6.74)		4.84 (2.92, 8.02)	
31-44	81% (294/353)	3.44 (2.28, 5.19)		4.13 (2.40, 7.10)	
45-72	89% (113/130)	6.65 (3.00,14.77)	**<0.0001**	7.74 (3.47, 17.27)	**<0.0001**
Sex					
Female	71% (561/770)	1		1	
Male	80% (646/801)	1.68 (1.26, 2.25)	**0.001**	1.72 (1.29, 2.30)	**0.001**
HIV status					
Negative	79% (813/1028)	1		1	
(+) treated	70% (71/103)	**0.64 (0.47, 0.87)**		**0.46 (0.30, 0.71)**	
(+) untreated	76% (10/13)	0.88 (0.22, 3.50)		0.55 (0.14, 2.16)	
(+) no treatment status	85% (73/85)	1.60 (0.82, 3.11)		1.22 (0.57, 2.26)	
Not tested	67% (240/342)	0.55 (0.31, 1.00)	**0.002**	1.05 (0.56, 1.95)	**0.002**
S. mansoni					
Uninfected	73% (676/915)	1		1	
Infected	80% (359/440)	1.55 (1.13, 2.11)	**0.008**	1.43 (1.04, 1.95)	**0.028**
N. americanus					
Uninfected	74% (927/1233)	1		1	
Infected	86% (106/120)	2.15 (1.18, 3.94)	**0.015**	1.55 (0.86, 2.80)	0.136
T. trichiura					
Uninfected	75% (924/1220)	1		1	
Infected	79% (111/135)	1.25 (0.66, 2.39)	0.480	1.60 (0.83, 3.08)	0.150
S. stercoralis					
Uninfected	74% (949/1255)	1		1	
Infected	85% (84/98)	1.82 (0.72, 4.62)	0.198	1.03 (0.41, 2.61)	0.947
Malaria					
Negative	75% (1142/1494)	1		1	
Positive	84% (50/60)	1.76 (0.81, 3.82)	0.144	3.49 (1.08, 11.28)	**0.038**

Seropositivity defined as reactivity to either ORF73 or K8.1 proteins. KSHV antibodies were detected using ELISA. Rapid tests were used to determine HIV status. Statistical analysis was performed using logistic regression, allowing for the survey design. *Schistosoma mansoni* and *Trichuris trichiura* infections were determined from a single stool sample using the Kato-Katz method. *Necator americanus* and *Strongyloides stercoralis* infections determined using PCR (polymerase chain reaction) method. [a]OR: odds ratios. [b]CI: Confidence Intervals. [c] adjusted for age, sex, HIV status, *S. mansoni*, *N. americanus* and malaria parasitaemia.

Although hookworm infection was positively associated with KSHV seropositivity in the unadjusted analysis (OR=2.15 (1.18, 3.94), p=0.015), this association was lost after adjusting for age group, sex, HIV serostatus, *S. mansoni* infection status and malaria infection status (Table 2). Helminth infections including *Trichuris trichiura* and *Strongyloides stercoralis* showed no association with KSHV seropositivity (Table 2). Other helminth

infections such as *Ascaris lumbricoides* and *Mansonella perstans* were not analysed using regression modelling due to the small numbers of infected participants.

Individuals with *S. mansoni* based on microscopy (aOR=1.43 (1.04, 1.95), p=0.028) or malaria parasite (aOR=3.49 (1.08, 11.28), p=0.038) infections were more likely to be KSHV seropositive (Table 2). The seropositivity of KSHV among individuals heavily infected with *S. mansoni* was 82% compared to 80% among lightly or moderately infected individuals, and 73% among uninfected individuals, although evidence for an increasing prevalence with increasing intensity was borderline after adjusting for possible confounders (P value for trend=0.068) (Table 3). We did not observe significant associations between *S. mansoni* detected by CCA or PCR and KSHV seropositivity (Supplementary Table 1). There was no effect of intensive versus standard anthelminthic treatment on either KSHV seropositivity or antibody levels (Table 4).

Table 3: Associations between KSHV seropositivity and *S. mansoni* infection intensity

Risk factor	KSHV seropositivity	Univariate		Age, sex, HIV, *N. americanus* and malaria adjusted	
		OR (95% CI)	P value	OR (95% CI)	P value
S. mansoni intensity					
Uninfected	73% (676/915)	1		1	
Light	80% (173/216)	1.53 (1.14, 2.05)		1.38 (0.98, 1.93)	
Moderate	80% (99/119)	1.48 (0.94, 2.33)	**0.049**	1.29 (0.79, 2.11)	0.068
Heavy	82% (87/105)	1.68 (0.71, 3.98)	trend	1.74 (0.69, 4.36)	trend

KSHV Seropositivity defined reactivity to either ORF73 or K8.1 proteins. KSHV antibodies detected using ELISA. OR: odds ratios. *Schistosoma mansoni* was determined from a single stool sample using the Kato-Katz method. Statistical analysis was performed using logistic regression, allowing for the survey design.

Table 4: Effect of anti-helminthic treatment on KSHV seropositivity and antibody levels

		KSHV seroprevalence				K8.1				ORF73			
	KSHV seroprevalence	Crude RR[a] (95% CI[b])	P value	Adjusted RR (95% CI)	P value	Crude diff[c] (95% CI)	P value	Adjusted diff (95% CI)	P value	Crude diff (95% CI)	P value	Adjusted diff (95% CI)	P value
Trial arm													
Standard	78% (550/710)	1		1		Ref		Ref		Ref		Ref	
Intensive	77% (657/861)	0.99 (0.92, 1.05)	0.690	1.00 (0.93, 1.07)	0.925	0.03 (-0.09, 0.15)	0.644	-0.06 (-0.18, 0.67)	0.352	0.06 (-0.06, 0.15)	0.312	-0.08 (-0.22, 0.06)	0.228

KSHV Seropositivity defined as reactivity to either ORF73 or K8.1 proteins. KSHV antibodies were detected using ELISA. [a]RR: risk ratio. [b]CI: Confidence Interval. [c]Diff: difference. Adjusted for sex, age group and HIV status.

Associations between KSHV seropositivity and *Schistosoma mansoni* antibody concentrations

IgE (n=364), IgG (n=372) and IgG4 (n=370) antibody concentrations against *S. mansoni* egg and adult worm antigens were measured in a subset of individuals with sufficient plasma for the analysis. Participants whose samples were used for this analysis were on average older than participants whose samples were not used; other participant characteristics were comparable (Supplementary Table 2). After adjusting for age group, sex and HIV status, increased levels of IgE to SWA (aOR=55.03 (3.14, 963.65), p=0.008) and SEA (aOR=8.20 (1.53, 44.05), p=0.016) as well as IgG to SWA (aOR=4.22 (0.98, 18.18) p=0.053) and SEA (aOR=2.57 (1.17, 5.68), p=0.02) were associated with an increased risk of being KSHV seropositive (Table 5).

Table 5: Associations between KSHV seropositivity and *Schistosoma mansoni* antibody concentrations

Antibody type	Univariate		Age, sex and HIV adjusted	
	OR (95% CI)	P value	OR (95% CI)	P value
IgE to SEA (n=364)	7.57 (1.54, 37.20)	**0.015**	8.20 (1.53, 44.05)	**0.016**
IgE to SWA (n=364)	83.03 (4.69, 1470.38)	**0.004**	55.03 (3.14, 963.65)	**0.008**
IgG to SEA (n=372)	3.03 (1.19, 7.77)	**0.023**	2.57 (1.17, 5.68)	**0.021**
IgG to SWA (n=372)	6.99 (1.24, 39.49)	**0.029**	4.22 (0.98, 18.18)	0.053
IgG4 to SEA (n=370)	1.30 (1.03, 1.62)	**0.026**	1.23 (0.96, 11.58)	0.097
IgG4 to SWA (n=370)	1.37 (0.96, 1.97)	0.080	1.18(0.82, 1.71)	0.362

KSHV Seropositivity defined reactivity to either ORF73 or K8.1 proteins. KSHV and *Schistosoma mansoni* antibodies detected using ELISA, measured in ng/mL and converted to μg/mL. OR: odds ratios per unit increase in antibody level. Statistical analysis was performed using logistic regression, allowing for the survey design. SEA: *Schistosoma mansoni* Egg Antigen; SWA: *Schistosoma mansoni* Worm antigen. Ig: Immunoglobuline

Discussion

KSHV prevalence can vary, even between geographically proximate areas [3]. We have previously reported a high KSHV prevalence of >95% in adults in the General Population Cohort in rural southwestern Uganda [5] But a lower prevalence of 61% amongst mothers in a peri-urban cohort [38]. This study shows high seropositivity of KSHV (>80% in 13+-year-olds) amongst Lake Victoria island communities with seropositive participants as young as one year. Additionally, we show that males were more likely to be KSHV seropositive compared to females. These findings are similar to those documented in other studies carried out in sub-Saharan Africa [4, 5, 39].

HIV prevalence in the studied communities was very high (17%). We observed a lower risk of being KSHV seropositive among individuals treated for HIV compared to HIV negative individuals. ART has been shown to lead to tumour regression among AIDS-KS patients [40-42]. This tumour regression has mainly been attributed to the reduction in the HIV load and the reconstitution of immune function [43]. Results from the current study, however, show an association between HIV treatment and lower KSHV prevalence. Additionally, others have shown a decline in KSHV viral load following HAART initiation [44, 45]. Since our study was cross-sectional however the interpretation of these data is difficult and the finding should be treated with caution.

The high untreated HIV prevalence coupled with other factors including parasite infections may contribute to the high prevalence of KSHV in this area. The burden of *S. mansoni* in these island communities is very high. Here we show that being infected with *S. mansoni* is associated with an increased risk of being KSHV seropositive. These results suggest a role for *S. mansoni* in KSHV transmission, which requires further investigation. Others have reported no association between *S. mansoni* and KSHV infections, possibly due to the low prevalence of KSHV or *S. mansoni* in the study areas [46, 47]. *In vitro* reactivation of the model gammaherpesvirus, MHV68 by *S. mansoni* was demonstrated by Reese *et al.,*

mediated through IL4 production [18]. Our human data are consistent with this model, as we observed an association with *S. mansoni* and KSHV seroprevalence. We also show that increasing IgE and IgG but not IgG4 antibodies to *S. mansoni* are associated with an increased risk of being KSHV seropositive. This would then suggest that *S. mansoni,* through IL-4 upregulation, may reactivate KSHV latently infected cells, or render KSHV uninfected individuals susceptible to infection, both of which can lead to increased transmission of the virus.

The association between *S. mansoni* and KSHV seropositivity was only observed if *S. mansoni* was detected using microscopy (KK) but not PCR or CCA, in this study. This might be attributed to the specificity and sensitivity of the three tests. KK might have detected only heavier infections compared to PCR, whereas CCA had very few negative samples for comparison. We did not see any effect of intensive versus standard anthelminthic treatment on KSHV seroprevalence, not surprisingly as such an intervention could only possibly affect incident but not the prevalent infection.

We also found malaria parasitemia to be associated with KSHV seroprevalence, consistent with our previous findings [4, 21], and these data reinforce the need to investigate the mechanism through which malaria infection impacts on KSHV and its subsequent role in KSHV transmission. Infection with *Plasmodium falciparum,* the main cause of malaria disease in Africa, induces inflammation which is normally regulated through induction of regulatory T cells and production of IL10 and TGF-β. Increased IL10 levels, a cytokine mainly produced by regulatory cells, has been reported in disseminated KS [48]. Plasmodia have also been shown to cause macrophage and dendritic cell dysfunction [49]. The immunosuppression caused by *P. falciparum* infection has also been shown to lead to the reactivation of some herpesviruses such as EBV, HSV-1 and VZV [50-54]. We, therefore, hypothesize that the immune dysregulation caused by malaria infection contributes to frequent reactivation of KSHV from latency. Since KSHV is transmitted by salivary exchange

[3, 55, 56], studies examining parasite co-infections and KSHV viral load in saliva are warranted.

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Chapter Five supplementary material

Supplementary Table 1: Association between KSHV seropositivity and *S. mansoni* infection detected using PCR and CCA method.

Risk factor	KSHV seroprevalence	Crude		Age, sex, HIV, *N. americanus* and malaria adjusted	
		OR (95% CI)	P value	OR (95% CI)	P value
S. mansoni (CCA)					
Uninfected	80% (160/205)	1		1	
Infected	76% (955/1225)	0.82 (0.50, 1.33)	0.402	0.87 (0.46, 1.65)	0.654
S.mansoni (PCR)					
Uninfected	73% (501/680)	1		1	
Infected	77% (532/673)	1.25 (0.97, 1.63)	0.083	1.16 (0.84, 1.62)	0.351

KSHV seropositivity defined reactivity to either ORF73 or K8.1 proteins. KSHV antibodies detected using ELISA. OR: odds ratios. PCR (polymerase chain reaction), CCA (circulating cathodic antigen). Statistical analysis was performed using logistic regression, allowing for the survey design.

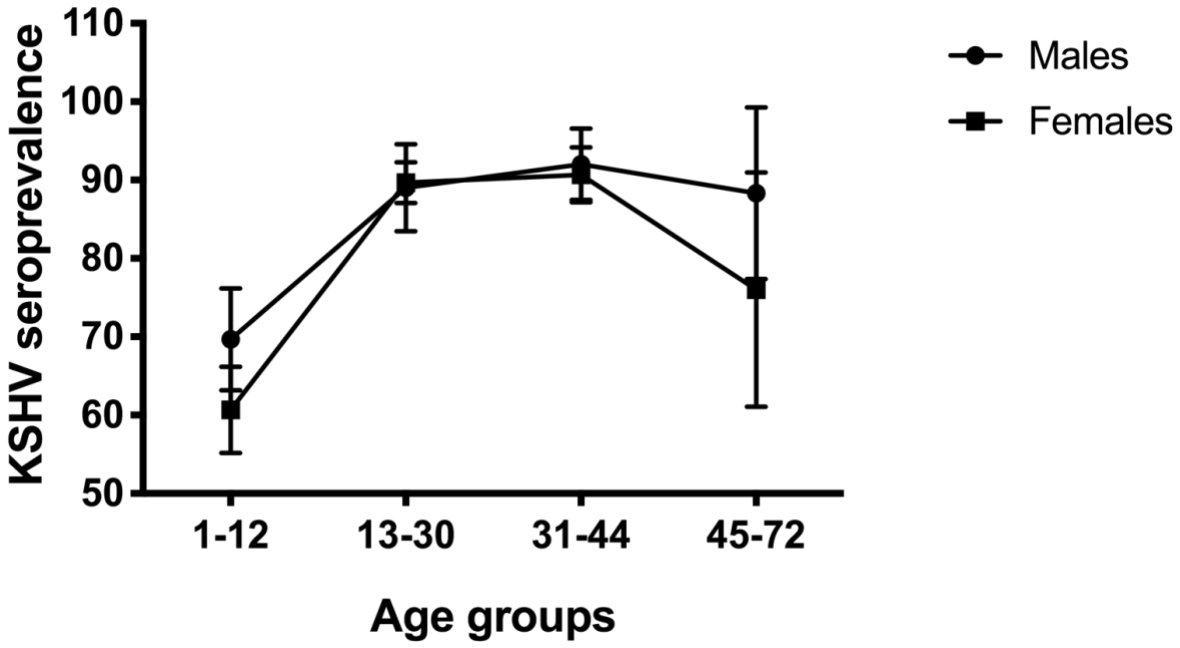
Supplementary Table 2: Infection status and study characteristics of participants tested for *Schistosoma mansoni* antibody responses compared to those not tested.

	Tested (n=372)	Not tested (n=1199)	P value
Age groups			
1-12	14%	36%	
13-30	52%	35%	
31-44	23%	22%	
45-72	11%	8%	<0.0001
Sex			
Male	44%	49%	0.053
HIV prevalence	17%	17%	0.914
Malaria parasitaemia	4%	4%	0.515
Schistosoma mansoni KK	16%	17%	0.858

P value obtained from a Chi^2 test, allowing for the survey design.

Chapter Five - additional results: KSHV seropositivity and associated risk factors at baseline, before anti-helminthic treatment

KSHV prevalence was high, at 70% among children aged 1-12 years and 93% among those aged 31 to 44 years (Figure 1), even prior to helminths treatment. HIV prevalence was 13%, while malaria parasitaemia was 14%. *S. mansoni* was the most prevalent helminth/parasite at 52% by Kato Katz (KK) and 72% by circulating cathodic antigen (CCA) (Table 1). Similar to what we observed after three years of helminths treatment, infection with *S. mansoni* (ascertained by KK) was associated with an increased KSHV prevalence (Table 2) and increased *S. mansoni* intensity was associated with an increased risk of being KSHV seropositive (Table 3). *S. mansoni* infection (detected by CCA) was not associated with KSHV seropositivity (Table 4).



Additional results Figure 1: KSHV seropositivity at baseline. KSHV seropositivity and 95% confidence intervals (CI) across ages 1 to 74 years. KSHV Seropositivity defined as reactivity to either ORF73 or K8.1 proteins. KSHV antibodies were detected using ELISA. Seropositivity and 95% CI were obtained in STATA, allowing for the survey design.

Additional results Table 1: Characteristics of the study population at baseline

Factor	**N=1310**
Age, median and interquartile range	25 (3 – 33)
Age group, years	
1-12	29% (362/1308)
13-30	40% (546/1308)
31-44	24% (319/1308)
45-74	7% (130/1308)
Sex (males)	56% (744/1310)
HIV prevalence	
Overall	13% (145/1150)
Participants aged 1-12 years	0.04% (1/288)
Participants aged 13-74 years	17% (144/862)
Malaria infection	
Overall	7% (92/1307)
Children (1-12 years)	14% (51/361)
Schistosoma mansoni prevalence	
KK	52% (606/1137)
CCA	72% (414/569)
Schistosoma mansoni intensity KK	
Uninfected	48% (531/1137)
Light infection	20% (238/1137)
Moderate	15% (183/1137)
Heavy	16% (185/1137)
Necator americanus prevalence PCR	26% (295/1136)
Hookworm prevalence KK	7% (82/1137)
Strongyloides stercoralis prevalence PCR	14% (176/1136)
Trichuris trichiura prevalence KK	11% (148/1137)
Ascaris lumbricoides prevalence KK	0.1% (15/1137)
Mansonella perstans prevalence	3% (37/1296)

Proportions were weighted to allow for the survey design. Helminths infection status and were determined from a single stool sample using Kato-Katz (KK) method or PCR (polymerase chain reaction) method or both. Rapid tests were used for HIV screening and microscopy was used for malaria diagnosis.

Additional results Table 2: Association between KSHV seropositivity and risk factors at baseline

Factor	KSHV prevalence	Crude OR[a] (95% CI[b])	P value	Adjusted[c] OR (95% CI)	P value
Age group (years)					
1-12	66% (236/362)	1		1	
13-30	90% (490/546)	4.65 (2.88,7.52)		5.14 (2.92, 9.05)	
31-44	93% (290/319)	6.50 (3.16, 13.36)		7.59 (3.31, 17.40)	
45-74	90% (74/81)	4.21 (1.41,12.57)	**<0.0001**	6.29 (2.54, 15.58)	**<0.0001**
Sex					
Female	81% (450/566)	1		1	
Male	86% (641/744)	1.41 (1.03, 1.94)	**0.035**	1.01 (0.63, 1.59)	0.980
HIV status					
Negative	84% (846/1005)	1		1	
Positive	89% (126/145)	0.65 (0.79, 3.45)	0.170	0.72 (0.36, 1.47)	0.357
S. mansoni					
Uninfected	77% (410/531)	1		1	
Infected	89% (533/606)	2.25 (1.45, 3.50)	**0.001**	1.86 (1.16, 2.99)	**0.012**
N. americanus					
Uninfected	82% (684/841)	1		1	
Infected	86% (258/295)	1.32 (0.92, 1.90)	0.125	1.21 (0.68, 2.15)	0.499
T. trichiura					
Uninfected	83% (816/989)	1			
Infected	86% (127/148)	1.28 (0.62, 2.67)	0.491		
S. stercoralis					
Uninfected	82% (786/960)	1		1	
Infected	89% (156/176)	1.77 (1.08, 2.89)	**0.025**	0.92 (0.59, 1.44)	0.708
Malaria					
Negative	84% (1016/1215)	1		1	
Positive	80% (72/92)	1.80 (0.51, 1.25)	0.310	1.27 (0.69, 2.33)	0.428

Seropositivity defined as reactivity to either ORF73 or K8.1 proteins. KSHV antibodies were detected using ELISA. Rapid tests were used to determine HIV status. Statistical analysis was performed using logistic regression, allowing for the survey design. *Schistosoma mansoni* and *Trichuris trichiura* infections were determined from a single stool sample using the Kato-Katz method. *Necator americanus* and *Strongyloides stercoralis* infections determined using PCR (polymerase chain reaction) method. [a]OR: odds ratios. [b]CI: Confidence Intervals. [c] adjusted for age, sex, HIV status, *S. mansoni*, *N. americanus* and malaria parasitaemia.

Additional results Table 3: Associations between KSHV seropositivity and *S. mansoni* infection intensity at baseline

Risk factor	KSHV seropositivity	Univariate		Age, sex, HIV, *N. americanus* and malaria adjusted	
		OR (95% CI)	P value	OR (95% CI)	P value
S. mansoni intensity					
Uninfected	77% (410/531)	1		1	
Light	87% (203/238)	1.91 (0.93, 3.92)		1.56 (0.79, 3.08)	
Moderate	91% (163/183)	3.00 (1.42, 6.37)	**<0.0001**	2.76 (1.17, 6.52)	**0.013**
Heavy	88% (167/185)	2.20 (1.27, 3.81)	trend	1.66 (0.84, 3.26)	trend

KSHV Seropositivity defined reactivity to either ORF73 or K8.1 proteins. KSHV antibodies detected using ELISA. OR: odds ratios. *Schistosoma mansoni* was determined from a single stool sample

using the Kato-Katz method. Statistical analysis was performed using logistic regression, allowing for the survey design.

Additional results Table 4: Association between KSHV seropositivity and *S. mansoni* infection detected using CCA method

Risk factor	KSHV seropositivity	Crude		Age, sex, HIV, *N. americanus* and malaria adjusted	
		OR (95% CI)	P value	OR (95% CI)	P value
S. mansoni (CCA)					
Uninfected	85% (132/155)	1		1	
Infected	84% (344/414)	0.92 (0.56, 1.51)	0.722	0.78 (0.38, 1.59)	0.401

KSHV seropositivity defined reactivity to either ORF73 or K8.1 proteins. KSHV antibodies detected using ELISA. OR: odds ratios. CCA (circulating cathodic antigen). Statistical analysis was performed using logistic regression, allowing for the survey design.

Chapter Six: Risk factors for Kaposi's sarcoma-associated herpesvirus (KSHV) DNA in blood and in saliva in rural Uganda

Preamble

Increased antibody levels are proxy measures of KSHV reactivation; viral detection in the blood is a more direct measure. Viral detection in the blood increases with KS disease progression and reduces with tumour regression. In this Chapter, we investigate the pattern of KSHV viral detection and levels of DNA in the blood and in saliva across a wide range of ages. This was carried out among apparently healthy individuals from rural Uganda where KSHV is highly prevalent. Similar data from comparable populations has not been published before. The following observations were made from this analysis:

(1) levels of KSHV DNA in blood did not correlate with levels of DNA in saliva
(2) viral detection in saliva is more frequent than viral detection in blood
(3) the frequency of individuals with detectable virus in blood and in saliva was highest among children
(4) increasing levels of KSHV DNA in the blood is associated with malaria parasitaemia.

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Student	Angela Nalwoga
Principal Supervisor	Stephen Cose
Thesis Title	Determinants of Kaposi's sarcoma associated herpesvirus seropositivity, viral DNA detection and cellular immune responses in Uganda

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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Where is the work intended to be published?	Plos Pathogens
Please list the paper's authors in the intended authorship order:	Angela Nalwoga, Marjorie Nakibuule, Vickie Marshall, Wendell Miley, Nazzarena Labo, Stephen Cose, Denise Whitby, Robert Newton
Stage of publication	**Submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	

Student Signature: [redacted] **Date:** 20th / May / 2019

Supervisor Signature: [redacted] Date: 21/05/2019

Risk factors for Kaposi's sarcoma associated herpesvirus (KSHV) DNA in blood and in saliva in rural Uganda

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Abstract

Detectable KSHV DNA in blood and increased antibody titres may indicate KSHV reactivation, while transmission of KSHV occurs via viral shedding in saliva. We investigated risk factors for KSHV DNA detection by real-time PCR, in blood and viral shedding in saliva, in 878 people aged 3 to 89 years of both sexes in a rural Ugandan population cohort. Helminths were detected using microscopy and malaria parasitaemia was identified using rapid diagnostic tests. Regression modelling was used for statistical analysis.

KSHV viral load in blood did not correlate with viral load in saliva, suggesting separate immunological control within each compartment. The proportion of individuals with detectable virus in blood was 23% among children aged 3-5 years, 22% among 6-12 years old, thereafter reducing with increasing age. The proportion of individuals with detectable virus in saliva increased from 30% in 3-5-year-old children to 45% in those aged 6-12 and decreasing subsequently with increasing age. Overall, 29% of males shed in saliva compared to 19% of females ($p = 0.008$). Together, these data suggest that young males may be responsible for much of the onward transmission of KSHV. Individuals with a current malaria infection had higher levels of viral DNA in the blood ($p = 0.031$) compared to malaria

uninfected individuals. This suggests that malaria may lead to KSHV reactivation, thereby increasing transmission and pathogenicity of the virus.

Keywords: Kaposi's sarcoma herpesvirus DNA, Risk factors, Uganda

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Author summary

Kapos's Sarcoma Herpesvirus (KSHV) is endemic in sub-Saharan Africa, though factors favouring its high transmission in the region are not well understood. Viral shedding in the saliva is associated with the transmission of the virus while viral detection in the blood is associated with reactivation and disease pathogenesis. Here we determine factors associated with both viral shedding in saliva and viral detection in blood in a KSHV endemic population. Our data show that children, particularly boys, may be a major source of viral transmission and malaria parasite infections could play a role in viral reactivation and susceptibility to infection.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) causes Kaposi's sarcoma (KS), multicentric Castleman disease (MCD) and primary effusion lymphoma (PEL) (1-6). Prevalence of KSHV and incidence of KS both vary geographically, (7-9) and are endemic in sub-Saharan Africa (10, 11)

Salivary exchange is the main route of transmission of KSHV, normally occurring in early childhood and increasing with age (12-15). In a rural population cohort in Uganda (the General Population Cohort – GPC), for instance, we have reported KSHV infections in children as young as one year (11, 16). In addition to viral shedding in saliva, viral DNA detection in blood and increased antibody titres to lytic antigens are markers of frequent KSHV reactivation (13). KSHV-associated oncogenesis and progression of diseases, as well as virus transmission, are all thought to be related to virus reactivation (17).

Viral DNA detection in blood has been associated with KS disease risk and progression (18-21). Additionally, treatment of AIDS-KS patients with cART has been shown to reduce KSHV load in blood to undetectable levels (18, 22). Determinants of KSHV DNA detection in blood among KSHV seropositive people in the general population is not well understood. Viral load in plasma and PBMCs has been reported mainly in high-risk groups such as HIV infected individuals and patients with KSHV related diseases (18, 23-27). A few studies have reported KSHV viral load in blood donors (adults) in non-endemic areas (28-30) and a study reported plasma viral load in a population-based HIV survey in an endemic area (31).

Environmental factors may contribute to high KSHV transmission in endemic areas. We have previously shown that KSHV seropositivity is associated with malaria parasitaemia (16), higher malaria antibody titres (32) and helminth infections (33, 34). We and others have reported that KSHV shedding in the saliva is more common in males compared to females (35, 36), but no study has investigated KSHV viral load in both blood and saliva in the same individuals within a population-based study in a KSHV endemic area.

This study investigated KSHV viral DNA detection in PBMCs and saliva in KSHV seropositive individuals aged 3 to 89 years from the General Population Cohort (GPC) – a longstanding cohort in rural south-western Uganda. We also determined the risk factors

associated with viral DNA detection and levels in PBMCs and in saliva and the relationship between viral DNA detection in PBMCs and saliva and KSHV antibody levels in plasma.

Methods

Study population and ethical approvals

This work was carried out within the General Population Cohort (GPC). The GPC is a community-based cohort of 22,000 people in 25 adjacent villages in south-western Uganda. It was established in 1989 to carry out HIV research; participants from the GPC have been followed ever since. Between July 2017 and November 2017, we nested a cross-sectional study within the GPC enrolling 975 KSHV seropositive (tested previously (32)), HIV negative individuals aged three to eighty-nine years. Participants were selected randomly after stratification for age, sex and household. Blood, stool and saliva samples were collected from these individuals. Peripheral blood mononuclear cells (PBMCs) and plasma were obtained from blood for immunological and virological analyses. Stool samples were used for helminth diagnosis while saliva was used for KSHV viral DNA detection and quantification. Socio-demographic data were collected using standard questionnaires. This study was approved by the UVRI-Research and Ethics Committee (REC) (reference number: GC/127/16/09/566), the Uganda National Council for Science and Technology (UNCST) (reference number: HS2123) and LSHTM Ethics Committee (reference number: 11881). Written informed consent was obtained from all adults aged 18 years and above. Parents or guardians consented for children below 18 years, additionally, children aged 8-17 years provided written assent.

Laboratory procedures

KSHV DNA was quantified in PBMCs and saliva from 878 KSHV seropositive individuals. A pellet of about two million PBMCs and saliva pellets were processed for DNA extraction. Study participants were instructed to rinse with 5mL of Listerine mouthwash, emptying it, as well as saliva in a falcon tube. Aliquots (of 1mL each) of saliva were spun at 13,000rcf for 10

minutes to form saliva pellets. Thereafter the supernatant was removed and the saliva pellet stored at -80^{o}C. Genomic DNA was extracted from PBMCs and saliva pellets using a QIAamp blood kit (Qiagen, Valencia, CA), following the manufacturer's instructions. KSHV DNA was quantified using real-time PCR, following procedures previously reported (13, 37, 38). KSHV DNA was detected using primers and a probe specific to the K6 gene region (39). Additionally, the number of cellular equivalents in PBMCs were determined using a quantitative assay specific to human endogenous retrovirus 3 (ERV-3) (39), which is present in two copies per genomic cell. Raw copies were reported for saliva KSHV DNA. Samples were amplified in triplicate; the samples that were positive in one or two reactions in the KSHV K6 assay were designated as qualitative positives.

Using an in house Luminex assay and ELISA, previously reported (40-42), plasma samples were tested for IgG antibody levels to the KSHV K8.1 (lytic) and ORF73 (latent) antigens. The ELISA was used to confirm serostatus while the Luminex assay was used to determine antibody levels, due to its wider dynamic range. Malaria parasitemia was diagnosed using rapid diagnostic tests (RDT) (ONE STEP Malaria HRP-II (P.f) and pLDH (Pan) Antigen Rapid Test). A single stool sample was provided by each participant. This was analysed for helminths (*Schistosoma mansoni*, *Ascaris lumbricoides*, *Tichuris trichiura*, *Trichostrongylus spp* and hookworm) using the Kato Katz microscopy method following the manufacturer's instructions. Details of this procedure have been reported elsewhere (43, 44).

Statistical analysis

Statistical analysis was carried out using STATA version 13 (StataCorp, College Station, Texas USA). Graphs were drawn using STATA and GraphPad Prism version 6. Qualitative positive samples were given a constant value of 0.04 for saliva and 0.5 for PBMCs, which were below the values of the lowest qualified samples for quantitative analysis. Viral load levels were $\log_{10}$ transformed. First, risk factors associated with viral DNA detection (as a categorical outcome variable) in saliva and blood, separately, were obtained using logistic

regression modelling. Thereafter, risk factors associated with increasing levels of viral DNA (as a continuous outcome variable) in saliva and in blood, separately, were determined using linear regression modelling. Likelihood ratio tests were used to select the best fit models.

Results

Study participants' characteristics

We tested 878 individuals for KSHV viral DNA; 49% (410/834) were males, while 3% (27/840), 11% (95/840), 13% (110/840), 8% (67/840), 17% (139/840), 14% (121/840), 14% (118/840), 9% (74/840) and 11% (89/840) were aged 3-5, 6-12, 13-18, 19-25, 26-35, 36-45, 46-55, 56-65 and 66-89 years respectively. The proportion of individuals with malaria parasitaemia was 4% (34/834) overall and 11% (13/120) among children 3-12 years. Previously we have reported an annual malaria prevalence of 18% in the same population (16). The lower prevalence of malaria infection in this study might be attributed to sample collection during the dry season. The prevalence of helminths was as follows: Hookworm was the most prevalent at 15% (104/686), followed by *Schistosoma mansoni* and *Ascaris lumbricoides* at 1% (8/686) each and *Trichuris trichiura* at 0.1% (1/686). We may have slightly underestimated the true prevalence of helminths, because a single, rather than triple sample test, was used.

Blood and saliva KSHV DNA detection and levels of viral DNA

We did not observe a correlation between KSHV DNA copy numbers in PBMCs and DNA copy numbers in saliva (Figure 1). The proportion of individuals with detectable viral DNA in saliva was higher than the proportion of people with the detectable viral DNA in PBMCs (Figure 2a and 2b). Children had the highest proportion of detectable viral DNA in PBMCs (Figure 2a) and in saliva (Figure 2b), decreasing with increasing age in adults. The trend was similar for females and males, with males having higher proportions of detectable viral DNA in saliva.

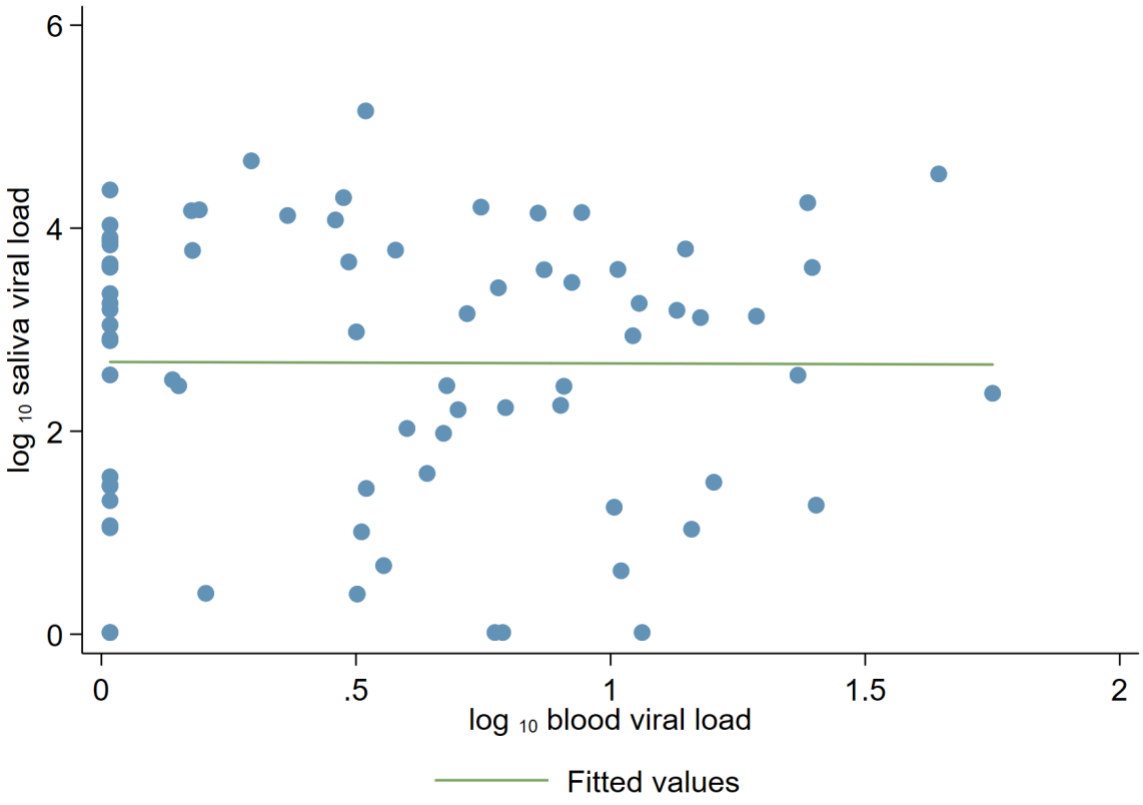

Figure1: KSHV viral load in saliva and in peripheral blood mononuclear cells (PBMCs). KSHV viral loads were measured using real time PCR.

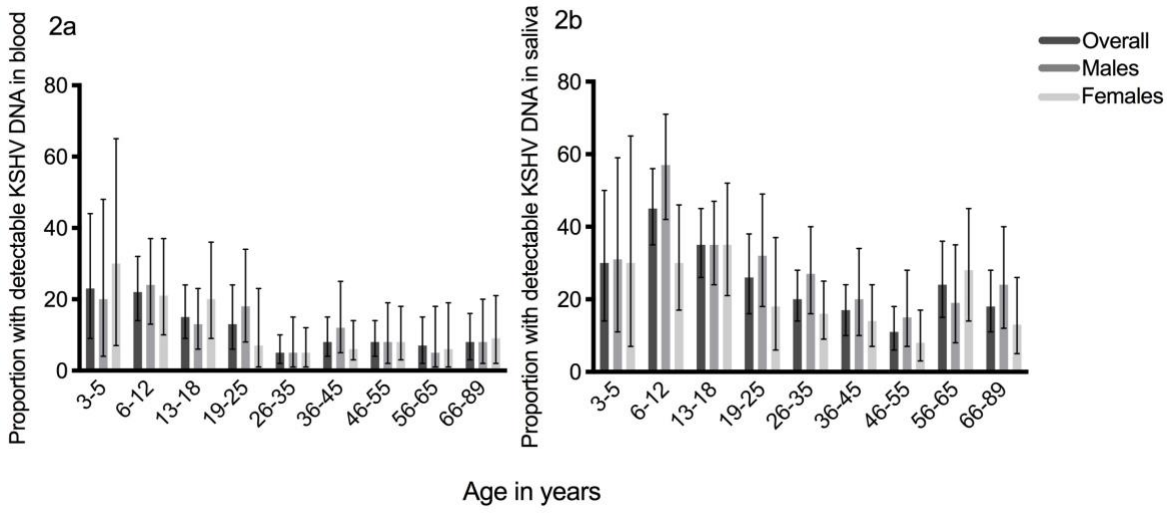

Figure 2: Proportion of individuals with detectable KSHV in peripheral blood mononuclear cells (PBMCs) (2a) and saliva (2b). KSHV viral loads were measured using real time PCR.

Associations between risk factors and KSHV DNA in PBMCs

The proportion of individuals with detectable viral DNA in PBMCs decreased with increasing age; this trend was significant even after adjusting for sex and parasite infections (Table 1). Individuals infected with malaria parasites had higher levels of KSHV DNA in blood compared to malaria uninfected individuals (adjusted regression coefficient 0.79 (0.07,1.50), p=0.031) (Table 2). We observe no statistically significant associations with other measured risk factors including age, sex, hookworm and *S. mansoni* infections (Table 2).

Table 1: Risk factors for the presence of detectable KSHV DNA in blood (categorically)

	% detectable viral DNA in blood	OR[a] (95% CI)	P value	Adjusted[b] OR (95% CI)	P value
Age group					
3-12	23% (27/120)	1		1	
13-25	15% (26/177)	0.59 (0.33, 1.08)		0.63 (0.33, 1.17)	
26-50	7% (20/307)	0.24 (0.13, 0.45)		0.29 (0.15, 0.57)	
50+	8% (18/227)	0.30 (0.16, 0.57)	<0.0001	0.34 (0.16, 0.72)	0.0014
Sex					
Female	10% (41/419)	1		1	
Male	12% (49/406)	1.27 (0.82, 1.96)	0.294	0.89 (0.55, 1.45)	0.638
Malaria parasiatemia					
Negative	10% (83/791)	1		1	
Positive	21% (7/34)	2.21 (0.93, 5.24)	0.071	1.59 (0.64, 3.95)	0.321
S. mansoni					
Negative	11% (77/672)	1		1	
Positive	43% (3/7)	5.80 (1.27, 26.38)	0.023	11.04 (2.16, 56.97)	0.004
Hookworm					
Negative	13% (74/576)	1		1	
Positive	6% (6/103)	0.42 (0.18, 0.99)	0.048	0.41 (0.16, 1.04)	0.061

[a] OR: odds ratio, [b] adjusted for age, sex, malaria parasitaemia, *S. mansoni* and hookworm infection status. Logistic regression was used for statistical analysis. Malaria parasitaemia was determined using rapid diagnostic tests (RDTs). Helminth status was determined from a single stool sample using the Kato-Katz method.

Table 2: Risk factors associated with increasing levels of KSHV DNA in blood (continuously)

	Coef[a]. (95% CI)	P value	Adjusted[b] Coef. (95% CI)	P value
Age group				
3-12	Ref		Ref	
13-25	-0.39 (-0.86, 0.07)		-0.35 (-0.84, 0.15)	
26-50	0.19 (-0.31, 0.69)		0.26 (-0.30, 0.83)	
50+	-0.32 (-0.83, 0.19)	0.084	-0.06 (-0.70, 0.57)	0.160
Sex				
Female	Ref		ref	
Male	0.10 (-0.27, 0.47)	0.594	0.21 (-0.19, 0.61)	0.292
Malaria parasitaemia				
Negative	Ref		Ref	
Positive	0.71 (0.05, 1.38)	0.036	0.79 (0.07, 1.50)	0.031
S. mansoni				
Negative	Ref		Ref	
Positive	0.17 (-0.87, 1.21)	0.750	-0.15 (-1.30, 1.00)	0.797
Hookworm				
Negative	Ref		Ref	
Positive	0.30 (-0.45, 1.05)	0.79	0.37 (-0.46, 1.21)	0.372

[a] Coef: linear regression coefficient, [b] adjusted for age, sex, malaria parasitaemia, *S. mansoni* and hookworm infection status. Linear regression modelling was performed on $\log_{10}$ transformed KSHV DNA levels for statistical analysis. Malaria parasitaemia was determined using rapid diagnostic tests (RDTs). Helminth status was determined from a single stool sample using the Kato Katz method.

Associations between risk factors and KSHV DNA in saliva

Overall, males had a higher risk of shedding viral DNA compared to females; adjusted Odds Ratio 1.63 (1.14, 2.34), p=0.008 (Table 3). Similar to PBMCs, the proportion of shedders in saliva diminished with increasing age, even after adjusting for sex and parasite infections p=0.0001 (Table 3). Additionally, compared to females, males had higher levels of KSHV DNA in saliva (adjusted regression coefficient 0.46 (0.05,0.87), p=0.027) (Table 4).

Table 3: Risk factors for the presence of detectable KSHV DNA in saliva (categorically)

	% detectable viral DNA in saliva	OR[a] (95% CI)	P value	Adjusted[b] OR (95% CI)	P value
Age group					
3-12	42% (50/120)	1			
13-25	31% (55/175)	0.64 (0.40, 1.40)		0.61 (0.37, 1.02)	
26-50	18% (56/310)	0.31 (0.19, 0.49)		0.38 (0.24, 0.63)	
50+	17% (39/231)	0.28 (0.18, 0.47)	<0.0001	0.30 (0.18, 0.54)	0.0001
Sex					
Female	19% (79/423)	1		1	
Male	29% (119/407)	1.80 (1.30, 2.49)	<0.0001	1.63 (1.14, 2.34)	0.008
Malaria parasitaemia					
Negative	24% (188/796)	1		1	
Positive	29% (10/34)	1.35 (0.63, 2.87)	0.439	0.98 (0.44, 2.16)	0.952
S. mansoni					
Negative	26% (174/675)	1		1	
positive	43% (3/70)	2.16 (0.48, 9.74)	0.317	2.43 (0.51, 11.52)	0.265
Hookworm					
Negative	27% (158/578)	1		1	
Positive	18% (19/104)	0.59 (0.35, 1.009)	0.054	0.66 (0.38, 1.14)	0.136

[a] OR: odds ratio, [b] adjusted for age, sex, malaria parasitaemia, *S. mansoni* and hookworm infection status. Logistic regression was used for statistical analysis. Malaria parasitaemia was determined using rapid diagnostic tests (RDTs). Helminth status was determined from a single stool sample using the Kato Katz method.

Table 4: Risk factors associated with levels of KSHV DNA in saliva (continuously)

	Coef.[a] (95% CI)	P value	Adjusted[b] Coef. (95% CI)	P value
Age group				
3-12	Ref		Ref	
13-25	-0.50 (-1.02, 0.21)		-0.61 (-0.13, -0.09)	
26-50	-0.31 (-0.82, 0.21)		-0.26 (-0.80, 0.28)	
50+	0.78 (-1.35, -0.21)	0.049	-0.76 (-1.40, -0.11)	0.048
Sex				
Female	Ref		Ref	
Male	0.51 (0.12, 0.89)	0.010	0.46 (0.05, 0.87)	0.027
Malaria parasitaemia				
Negative	Ref		Ref	
Positive	0.20 (-0.67, 1.07)	0.651	0.05 (-0.82, 0.92)	0.909
S. mansoni				
Negative	Ref		Ref	
positive	-0.58 (-2.12, 0.96)	0.460	-0.38 (-1.94, 1.18)	0.635
Hookworm				
Negative	Ref		Ref	
Positive	-0.36 (-0.96, 0.32)	0.326	-0.37 (-1.03, 0.29)	0.270

[a] Coef: linear regression coefficient, [b] adjusted for age, sex, malaria parasitaemia, *S. mansoni* and hookworm infection status. Linear regression modelling was performed on $\log_{10}$ transformed KSHV DNA levels for statistical analysis. Malaria parasitaemia was determined using rapid diagnostic tests (RDTs). Helminth status was determined from a single stool sample using the Kato Katz method.

KSHV DNA detection and antibody levels

Individuals with detectable viral DNA in PBMCs (Figure 3a) and in saliva (Figure 3b) had higher IgG antibodies to the K8.1 antigen ($p<0.0001$), as previously reported (13). There was no difference in IgG antibodies to ORF73 antigen between individuals with or without detectable viral DNA in the blood (Figure 3c) or in saliva (Figure 3d).

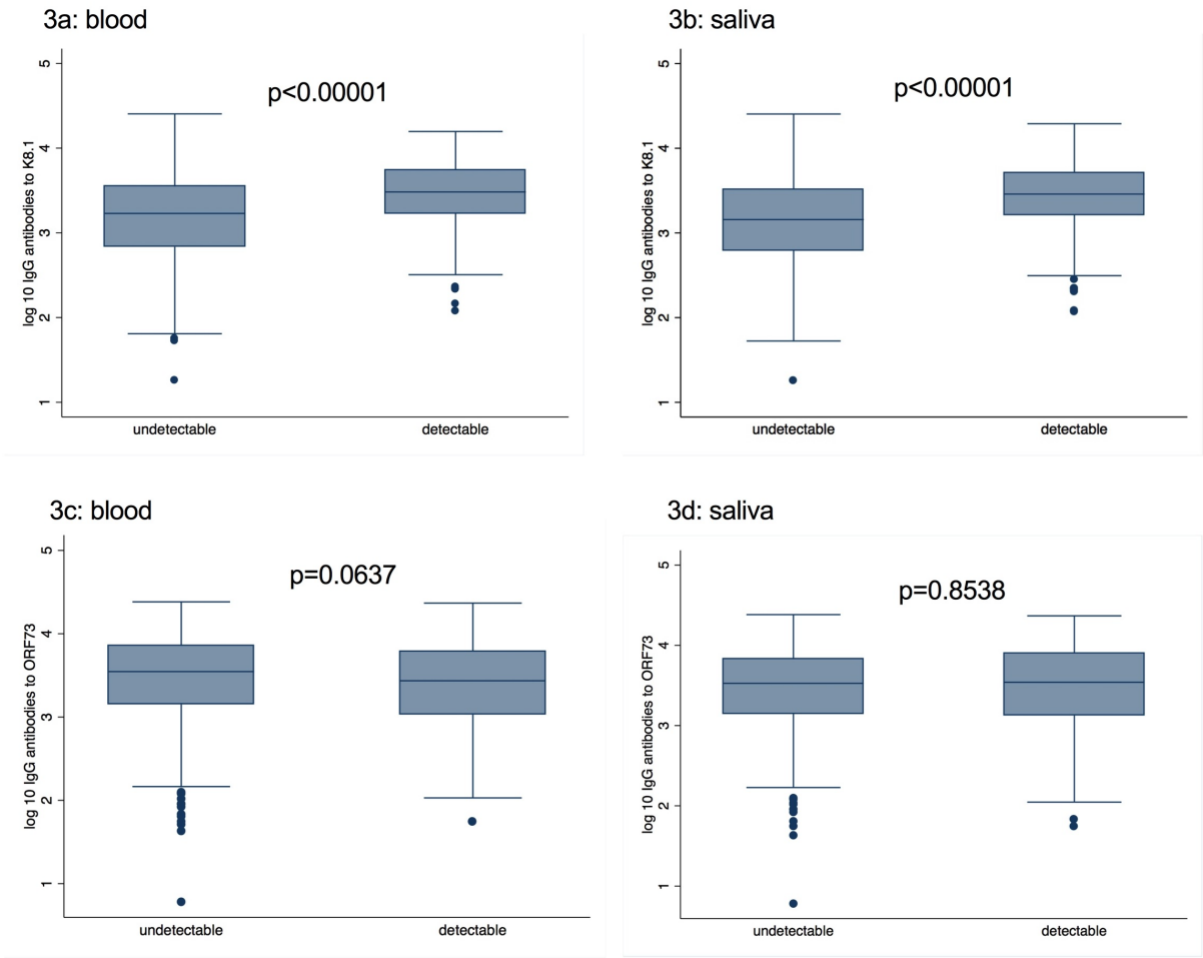

Figure 3: IgG antibody levels to K8.1 (a & b) and ORF73 (c & d) protein among individuals with and without detectable KSHV in the blood (a & c) and in saliva (b & d). Antibodies were measured using the Luminex assay. These antibodies were log_{10} transformed. P values were obtained from a ttest after log_{10} transformation of the data. The box show all values within the 25^{th} and 75^{th} quatiles (interquatile range). The line in the box represent the median. The whiskers are +/- 2 to the interquatile range. The dots represent outliers.

Discussion

This is the first population-based study to report on presence and levels of KSHV viral DNA in blood and saliva in apparently healthy people across the life-course. The proportion of individuals with detectable viral DNA in saliva was higher than the proportion of individuals with detectable viral DNA in blood, consistent with previous reports (22, 45-47). We have previously reported KSHV and EBV DNA shedding in the saliva of children and their mothers in Uganda and noted that EBV DNA was shed more frequently and at higher levels than KSHV (38).

In this study, we observed no correlation between levels of KSHV DNA in blood and in saliva. Detection of KSHV DNA in blood and in saliva may reflect reactivation of the virus or an initial infection that manifests with lytic replication. Viral shedding in saliva leads to the transmission of the virus (13), while viral load in blood has been implicated in disease risk and progression (18, 23). The lack of a correlation between blood and saliva viral DNA suggests that the mechanisms for reactivation of the virus in blood and in saliva may be different. This may imply that distinct immune control measures are required to prevent viral reactivation in different compartments. Studies of immune correlates of KSHV DNA detection in saliva and PBMC are therefore warranted.

The proportion of KSHV DNA detected in blood and saliva was highest in children, compared to adults. Previous studies have reported a high risk of KSHV seropositivity in children born to KSHV seropositive mothers (13, 35, 48). However, seropositivity in children whose mothers were seronegative has also been reported (48, 49). Our current study suggests that siblings or playmates may also be a major source of transmission to uninfected children.

High viral load among children could be associated with co-infections that are very prevalent in childhood such as malaria; we also observed the highest prevalence of malaria

parasitaemia in the same age group. We showed that participants with malaria parasitaemia have higher levels of KSHV DNA in blood compared to those uninfected with malaria. This is the first study to relate malaria parasitaemia directly with KSHV load in blood. We have previously reported associations between malaria (parasitaemia and antibodies) and KSHV seropositivity (16, 32-34). Results from the current study support the potential role of malaria in KSHV pathogenesis. The mechanisms for the association between malaria and KSHV viral load could include immunomodulation and dysfunction associated with repeated malaria infections (50, 51).

In the present study, males (both men and boys) were more likely to shed KSHV DNA in saliva and had higher levels of viral DNA compared to females. This is consistent with previous studies by us and others (31, 36, 38). Sex differences in immune control of KSHV infection might contribute to the higher risk of KS in men (52, 53). These findings warrant further study.

We have previously reported that high KSHV K8.1 antibody titres are associated with and predictive of KS risk (54). In the current study, we have observed that people with detectable viral DNA in both blood and saliva have higher IgG antibody levels for K8.1 but not ORF73, compared to individuals without the detectable viral DNA. This association confirms our previous hypothesis (54) that increased lytic antibody levels reflect more frequent KSHV reactivation.

In summary, our data are consistent with high rates of KSHV transmission in rural Uganda. This might be partly attributed to parasite co-infections such as malaria which interfere with immune control, or make uninfected children susceptible to infections. Studies investigating the mechanism through which malaria affects KSHV are required. Additionally, characterisation of protective immune responses to KSHV is needed to inform vaccine development and to develop strategies to lower KSHV transmission in endemic areas.

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Chapter Seven: IFN-γ cellular responses to the KSHV proteome in individuals from rural Uganda

Preamble

Cellular immune responses to KSHV have been poorly studied and published reports rarely replicate previous findings. In the current study, we used 82 peptide pools spanning the entire KSHV proteome to determine cellular immune responses and how they relate to viral detection in PBMCs. Our data show no immune dominance and a robust response among adults who control the virus in the blood. These results require a bigger sample size for confirmation. Therefore we will test more samples before submitting the manuscript for publication.

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SECTION A – Student Details

Student	Angela Nalwoga
Principal Supervisor	Stephen Cose
Thesis Title	Determinants of Kaposi's sarcoma associated herpesvirus seropositivity, viral DNA detection and cellular immune responses in Uganda

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
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Where is the work intended to be published?	Blood
Please list the paper's authors in the intended authorship order:	Angela Nalwoga, Romin Roshan, Marjorie Nakibuule, Vickie Marshall, Wendell Miley, Nazzarena Labo, Stephen Cose, Robert Newton, Denise Whitby
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	

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Supervisor Signature: [redacted] Date: 21/05/2019

Kaposi's sarcoma-associated herpesvirus (KSHV) specific IFN-γ cellular immune responses in relation to age and viral DNA detection in healthy individuals from rural Uganda

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Abstract

Little is known about the cellular immune response to KSHV among apparently healthy KSHV seropositive individuals from endemic areas. We investigated IFN-γ responses to the entire KSHV proteome in individuals from a rural Ugandan population cohort. An *ex-vivo* IFN-γ ELISpot assay was carried out on PBMCs from 34 individuals, using 82 KSHV ORFs. KSHV viral load in PBMC was determined using real-time PCR.

Cellular immune responses to KSHV were heterogenous showing no shared immunodominance. The responses were most intense in young adults (27-44 years old), lower in older people and very low in children. Among young adults, those without robust cellular immune responses had detectable viral DNA in their blood, whereas those with a good response had no evidence of viral DNA in blood.

Although the sample size in the current study was small, these findings suggest that cellular immune responses decline with age and robust IFN-γ responses correlate with a lack of detectable KSHV viral load in blood.

Keywords: Kaposi's sarcoma herpesvirus, cellular responses, Uganda

Introduction

The adaptive immune response to KSHV is central in protection from KSHV associated malignancies (Kaposi's sarcoma-KS, Multicentric Castleman's disease-MCD and primary effusion lymphoma-PEL) [1, 2]. This is shown by the substantial increase in the risk of developing KS and other KSHV malignancies in immunocompromised individuals due to HIV or immunosuppressive drugs [3, 4]. Furthermore, the pivotal role of T cells in controlling latent herpesviruses, including EBV, CMV and HSV, has been documented [5, 6], although similar evidence for KSHV is still lacking.

During the adaptive immune response, CD4+ T cells help B cells and CD8+ T cells to function efficiently; cytotoxic CD8+ T cells kill virally-infected cells and B cells produce antibodies which neutralise and opsonise viruses, as well as activating complement to lyse extracellular virus. A protective role of antibody responses in KSHV pathogenesis and disease progression has not been observed [4], and few studies have investigated CD4+ T cells in relation to KSHV and its associated malignancies [2]. Conversely, several studies have reported a predominant role of cytotoxic CD8 T cells in the control of Classic, Iatrogenic and AIDS-related KS [1, 2, 7, 8]. Only two studies have reported cellular responses to KSHV in sub-Saharan Africa [9, 10].

A higher frequency of T cells (mainly CD8+) has been reported in KS patients who achieve complete remission compared to cases with unresolved KS [11-13]. In comparison to KS patients, higher T cell responses and frequency in asymptomatic individuals have been reported [14]. Treatment with highly active antiretroviral therapy (HAART) of AIDS-KS patients has been associated with an improved T cell response, correlating with KS regression [15, 16].

KSHV encodes over 80 proteins [17]. This has made the search for a T cell immunodominant peptide extremely difficult. Previous studies have each used different

peptides, making each study unique, but difficult to compare between studies. A systematic approach of using the entire KSHV proteome to investigate KSHV antibody responses in Americans revealed additional immunogenic KSHV proteins than had previously been reported [18]. Using a similar approach, the same authors investigated T cell responses to the entire KSHV proteome among Americans [19]. This study confirms T cell immune variability and the lack of an immune dominant peptide in that population.

As noted above, all of the T cell studies have been done among patients with some form of KSHV associated malignancy and, in individuals from countries where KSHV is not endemic. T cells studies in healthy individuals from endemic countries are still lacking. Due to the high transmission of KSHV and the high burden of KS in endemic areas, understanding T cell responses in these populations is paramount in the development of a KSHV vaccine for these key populations. Vaccination would halt transmission and prevent KSHV-associated cancers.

We undertook the same systematic approach to investigate T cell responses to the entire KSHV proteome in a rural cohort in Uganda, where KSHV is endemic. We focused on age-specific responses among KSHV seropositive individuals with and without KSHV DNA in PBMCs.

Methods

Study population

The General Population Cohort (GPC) is a community-based cohort of 22,000 people in 25 adjacent villages [20, 21]. Located in southwestern Uganda, the GPC is a typical Ugandan rural community. Previously, we have documented high seropositivity of KSHV in the GPC [22, 23]. For this study, we recruited individuals who were KSHV seropositive, but HIV seronegative from the GPC in 2017, to investigate determinants of KSHV viral detection. Only one individual was recruited per household. Blood samples were processed to isolate

PBMCs (stored in liquid nitrogen for later immunological assays) and plasma (stored at -80 °C for later use). Cell pellets containing two million PBMCs were stored at -80°C for later DNA extraction. KSHV-specific IFN-γ responses were determined in 34 participants aged six to 87 years. Purposive sampling was used ensuring that participants with and without detectable viral DNA in blood from each age group were selected. Details of the characteristics of the participants selected are shown in Supplementary Table 1.

KSHV ELISPOT assay

Overlapping 15mer peptides were synthesised to span the entire KSHV proteome, and peptides divided into pools representing each open reading frame (ORF) [19]. Using these peptide pools, working concentrations of 5μg/ml/peptide were prepared in 96 well culture plates using AIM-V media. The MABTECH Human IFN-γ ELISpot kit (Code: 3420-2APT-10) was used for the assay, with a few alterations to the manufacturer's protocol. The ELISPOT plates with the capture antibody from the kit were washed 5 times with 200μl of 1xPBS per well. Afterwards, thawed cells were added to the plates in a volume of 100μl containing 150,000 cells per well. The plates were covered with the lid and wrapped in aluminium foil and transferred to a 5% CO_2 37°C incubator for a 24 hour resting period. To stimulate them, 100μl per well of the peptide pools were added to the cells. Anti-CD3 and a pool of peptides from flu, CMV and EBV were added as positive controls in triplicate. Media and a non-human antigen (Simian immunodeficiency virus (SIV) peptides) were also added as negative controls in triplicate. The plates were then incubated at 37°C for 46-48 hours. Following stimulation, cells were washed 5 times with 200μl of PBS per well and 100μl of anti-human IFNγ IgG conjugated to alkaline phosphatase (Code: 7-B6-ALP) was added at a dilution of 1/6 in PBS + 0.5% FBS. The plates were incubated at room temperature (25°C) for 2 hours. After the incubation, the plates were washed 5 times with 200μl of 1xPBS per well and 100μl of filtered 5-bromo-4-chromo-3-indolyl-phosphate (BCIP)/nitroblue tetrazolium (NBT)-plus substrate (Code: 3650-10) added per well. The plates were then incubated at room

temperature for 7 minutes and the reaction stopped by washing the plate with running tap water. The plates were dried in the dark overnight, subsequently, the spots were counted using an ELISPOT reader (AID ELISPOT Reader HR model: ELHR010307005). This protocol has been reported elsewhere [19].

KSHV ELISA and PCR

Plasma samples from the same individuals were tested for KSHV IgG antibodies to K8.1 and ORF73 antigens to confirm seropositivity using methods previously reported [24, 25]. DNA extracted from 2 million PBMC pellets was tested for KSHV load using real-time PCR as previously reported [26-28].

Statistical analysis

Graphical representation was done using GraphPad Prism 8.0.1 (145). Spot Forming Cells (SFC) per million PBMC were calculated from the number of spots counted by the ELISpot reader and the number of PBMCs plated using Microsoft Office Excel (version 15.23). A cut-off of 60 SFC derived from the mean SFC of the SIV negative control and its standard deviation was applied. Therefore, individuals with SFC below 60 were considered non-responders.

Ethical approvals

This study was approved by the UVRI-Research and Ethics Committee (REC) (reference number: GC/127/16/09/566), the Uganda National Council for Science and Technology (UNCST) (reference number: HS2123) and the LSHTM Ethics Committee (reference number: 11881). Written informed consent was obtained from all adults aged 18 years and above. Children below 18 years were consented into the study by their parents or guardian; we also sought, in addition to parental consent, written assent from children aged between 8-17 years.

Results

KSHV specific IFNγ responses

IFNγ responses to 84 peptide pools were determined in PBMC from 34 individuals. SFCs per million PBMCs to peptide pools raged from 0 to 3873, while those to CEF (a cocktail of peptides from CMV, influenza and EBV) ranged from 0 to 392. All the samples responded to anti-CD3 while about half of the samples responded to CEF. Responses to CEF were independent of responses to KSHV peptides (Figure 1).

There was no consistent pattern in response to peptide pools. Of the 84 peptide pools used, no reaction was observed to 26 (K12, ORF16, ORF19, ORF22, ORF23, ORF25, ORF26, ORF29a, ORF29b, ORF31, ORF35, ORF47, ORF48, ORF49, ORF53, ORF54, ORF56, ORF57, ORF60, ORF62, ORF64/1, ORF64/3, ORF70, ORF72, ORF74 and ORF75) in any individual, reactions to 35 were observed (K2, K4, K5, K6, K7, K9, K10, K14, K15, ORF2, ORF10, ORF11, ORF17, ORF18, ORF28, 0RF32, ORF33, ORF34, ORF36, ORF38, ORF39, ORF40, ORF43, ORF45, ORF46, ORF52, ORF55, ORF58, ORF59, ORF63, ORF64/2, ORF65, ORF66, ORF67 and ORF69) in one individual, while reactions to 23 were observed (K1, K3, K8, K11, K13/ORF71, ORF4, ORF6, ORF7, ORF8, ORF9, ORF20, ORF21, ORF24, ORF27, ORF30, ORF37, ORF41, ORF42, ORF44, ORF50, ORF61, ORF68 and ORF73) in more than one individual.

In regards to the participants selected, the majority (65%; 22/34) responded to at least one peptide pool. The individual who responded to the largest number of peptide pools belonged to the 27-44 age group and had the highest response overall as well. This individual responded to 27 peptide pools with the highest response of 3873 SFCs to K4 (Figure 1). In the 6-13 and 51-87 age groups, the individuals who responded to the maximum number of peptide pools reacted to 3 and 18 peptide pools respectively. The maximum number of people reacting to a single peptide pool (K11) was six (18%). Responses varied according to

age. Children aged 6 to 13 years had the lowest responses, ranging from 67-947 SFC, compared to young adults aged 27 to 44 years (60-3873SFCs) and older individuals aged 51-87 years (60-2140SFC). Additionally, only 40% (6/15) of children responded to at least one single peptide pool while 87% (13/15) of older individuals and 50% (2/4) of young adults responded to at least one peptide pool.

Generally, individuals responded to various KSHV peptide pools, without any immunodominance. The intensity and robustness of responses were highest in young adults (27-44 years), lower in older people (51-87) and very low in children (6-13 years). Among young adults, those without detectable virus had more robust responses compared to those with the virus in the blood (Figure 1).

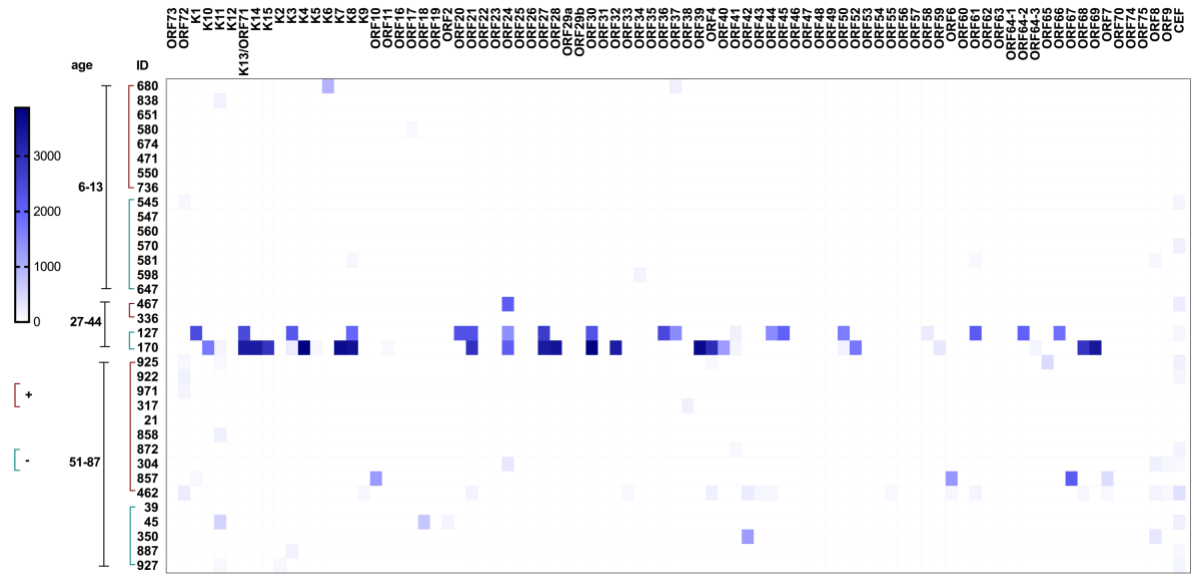

Figure 1: A heat map summarizing KSHV specific IFN-γ responses to 84 peptide pools in 34 individuals aged six to eighty-seven. The ELISpot assay was used to determine the responses. Spot forming cells (SFCs) per million PBMCs were recorded for each reaction. The intensity of the blue colour correlates with the number of SFCs per million PBMCs. The Y-axis labels show the study participant's identification numbers and the age band for each participant. The X-axis label shows the 84 peptide pools used in the analysis. (+) individuals with detectable KSHV DNA in blood. (-) individuals without detectable KSHV DNA in blood. GraphPad Prism version 8 was used to draw the heat map.

Discussion and conclusion

This is the first study to demonstrate KSHV cellular responses to the entire proteome among healthy individuals, of a wide age range, and from an endemic area. Interestingly, the responses we have observed in a KSHV-endemic population confirm the observations reported in US healthy donors and patients with KSHV related disease [19, 29], namely that there is a complete lack of immune dominance. Additionally, we have shown that the presence of a robust cellular response among young adults is associated with viral control, data that has not previously been demonstrated. We further showed that young adults have the highest and most robust cellular responses compared to children and older individuals and that older people compared to children have more robust and higher cellular immune response. We have previously reported that children have the highest rates of detection of KSHV DNA in PBMC. Taken together with the current study, we speculate that these observations are consistent with (presumably) recently infected participants failing to mount effective cellular immune responses favouring KSHV reactivation.

Previous studies [1, 2] have focused on only a few KSHV antigens to investigate cellular responses, with the exception of one study [19] which was carried out among Americans. Some of these studies showed that cellular responses are skewed to the latent, early lytic and late lytic antigens. In the current study, we confirm the previous findings in a population with high KSHV prevalence showing heterogenous responses across the whole proteome without any immune dominance.

Several studies have correlated KS regression and remission with improved T cell responses [11, 13, 14, 30, 31]. It has therefore been hypothesized that improving the T cell response in these patients provides immunity to both KS and KSHV, leading to tumour regression. Our study confirms the role of the cellular response in the control of KSHV reactivation. It is the first study to show KSHV control attributed to cellular responses in healthy individuals from an endemic area.

Young children and older individuals are at risk of KS in endemic areas [32], and infection with KSHV occurs in childhood [27, 33]. Children may be at risk of developing KS due to the immaturity of their developing immune system, and older people are likely more susceptible due to their ageing immune system (immunosenescence) and chronic inflammation [34]. Consistent with these hypotheses, it is not surprising then that our study showed that cellular responses to KSHV are weaker in these age groups, compared to young adults whom we show have a robust T cell response.

In summary cellular immune response to KSHV is likely paramount in the control of reactivation of the virus. KSHV does not illicit an immunodominant response. This has now been shown in two geographically distinct locations. This has major implications for vaccine design. Vaccine efforts may need to concentrate on multiple epitope vaccines, or attenuated whole virus, to induce effective protective immunity. Additionally, therapeutic provoking a specific immune response (shown to be protective) in individuals at a high risk of developing KS and other KSHV-associated malignancies is another possibility. Vaccination and therapeutic prevention would drastically reduce KS and other KSHV-associated malignancies which have a very poor prognosis in developing countries.

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Chapter Seven supplementary material

Supplementary Table 1: Study participants' characteristics

KSHV T cell responses (ELISpot) n=34	The proportion with detectable KSHV in PBMC[a]
Sex	
Male	61% (11/18)
Female	56% (9/16)
Age group	
1-13	53% (8/15)
27-44	50% (2/4)
51-87	67% (10/15)

[a]PBMC: peripheral blood mononuclear cells. KSHV viral load measured using real time polymerase chain reaction (PCR).

Chapter Eight: General discussion

Research aims, hypotheses and summary of findings

The prevalence of KSHV varies geographically [1, 2]. Sub-Saharan Africa bears the highest burden of the associated disease (KS) as well as of the infection (KSHV) [3, 4]. Currently, KSHV cannot be eradicated and, long-term remission has been achieved only in some KS cases [5]. KSHV infection is necessary, but not sufficient for oncogenesis and important co-factors, such as immunosuppression, have been identified [6]. In addition, the prognosis for those diagnosed with KS is very poor in sub-Saharan Africa [5]. To prevent disease, it is essential to control KSHV transmission and to identify people at risk of developing KS among those already infected. This PhD study had the following aims:

(1) To document the burden of KSHV in recent years in rural Uganda and Lake Victoria island fishing communities
(2) To use two markers of KSHV reactivation (increased antibody levels and viral load) to identify biological and other risk factors associated with KSHV reactivation
(3) To assess factors associated with transmission (using viral shedding in saliva as a proxy marker) in Uganda, where KSHV is endemic
(4) To investigate cellular immune responses to KSHV and its relationship to viral reactivation or control.

We hypothesized the following:

(1) That there would be urban-rural differences in prevalence, with higher prevalence in rural areas due to increased parasite burden and other KSHV risk factors
(2) That in the Lake Victoria Island communities, KSHV prevalence would be high due to the high parasite and HIV burden [9]

(3) That, based on previous findings [10-12], malaria, anaemia, and helminths would be associated with KSHV-specific antibody levels, as well as viral DNA detection in blood and saliva
(4) That malaria and helminths affect KSHV reactivation through anaemia, which leads to hypoxia
(5) That age of infection with KSHV affects viral control (based on findings relating to EBV infection) [13, 14]
(6) That KSHV DNA shedding in saliva and viral DNA detection in the blood would be high in both children and older people since these age groups are at higher risk of KS [15]
(7) That, similar to CMV [16], KSHV has cellular immune dominance that may be population specific
(8) That robust cellular, as well as antibody responses, would be associated with KSHV control in blood.

The following observations were made from three populations: the peri-urban population (EMaBS), Lake Victoria fishing villages (LaVIISWA) and rural Uganda population (GPC). KSHV was highly prevalent both in rural Uganda and Lake Victoria fishing villages, higher than the prevalence previously reported in a peri-urban population of Entebbe (EMaBS) [10-12]. In rural Uganda and Lake Victoria fishing villages, KSHV antibody levels and seropositivity generally increased with increasing age and KSHV seropositivity was higher in males compared to females. In the EMaBS, younger primary age of infection was associated with higher antibody levels in later years. These higher antibody levels were likely maintained because they were still high several years after seroconversion.

Schistosoma mansoni infection (which is highly prevalent in areas around water bodies with snails) was associated with KSHV seropositivity in fishing villages of Lake Victoria. Increase in IgE and IgG antibody concentrations to *S. mansoni* was associated with an increased risk

of being KSHV seropositive in island individuals. HIV treatment was associated with lower KSHV prevalence in the same communities.

In the GPC, malaria parasite infection was a risk factor for KSHV seropositivity and people infected with malaria tended to have higher KSHV antibody levels. The malaria association with KSHV seropositivity was also observed in LaVIISWA. Furthermore, malaria parasite-infected individuals had higher levels of KSHV DNA in PBMCs compared to malaria uninfected individuals. Lower haemoglobin levels (independent of malaria parasite infection) were associated with higher KSHV seropositivity and KSHV-specific antibody levels. Among seropositive individuals from rural Uganda, the proportion of individuals with detectable viral DNA in PBMCs and in saliva was highest in children and decreased as individuals got older. KSHV viral load was detected more often and at higher levels in saliva compared to blood in rural Uganda. Furthermore, levels of viral DNA in PBMCs did not correlate with those in saliva. IgG antibody levels to K8.1 recombinant protein were higher in individuals with detectable KSHV DNA in blood and in saliva. Male individuals were more likely to shed KSHV in saliva and had higher levels of viral DNA in saliva compared to females. In relation to the IFN-γ cellular response to 82 KSHV peptide pools, there was heterogeneity of response and no immune dominant phenotype was observed. Viral control (measured by viral DNA in PBMCs by PCR) was associated with age group-specific robust cellular response. Although cellular responses to KSHV increased as children became adults, these responses seemed to reduce in old age.

Discussion and conclusions

KSHV transmission in endemic areas may be affected by social behaviours leading to salivary exchange as well as factors affecting the immune system. Here we have focused on factors that may affect the immune system because KSHV activation has been shown to be sensitive to immune dysfunction [17]. Effects on adaptive immunity may increase the

reactivation of the virus, affecting viral control. This could lead to pathogenesis and increased transmission. Factors which affect innate immunity may also render uninfected individuals susceptible to infection, by preventing primary control of the infection.

We observed an increased risk of KSHV seropositivity if individuals were infected with malaria parasites. Although malaria parasite infection causes polyclonal non-specific B cell activation [18, 19], which could lead to increased antibody responses and explain the associations with antibody levels, we also observed increased levels of viral DNA in blood if infected with malaria (as measured by the presence of parasitaemia). Infection with malaria parasites has been shown to cause immune dysfunction of both the adaptive and the innate immune systems. Dendritic cell frequency and function are impaired by malaria infection as well as T and B cell function [20-22].

Similarly, we observed an increased risk of seropsitivity in people infected with *S. mansoni.* The adaptive immune effects of *S. mansoni* may affect KSHV reactivation. *S. mansoni* skews the immune response to a (Th) 2 type response characterized by the production of IgE antibody, eosinophilia and production of cytokines such as IL4 and IL5. It is also known to induce IL10 production, which is an immunomodulating cytokine. The (Th) 2 and regulatory immune responses elicited by *S. mansoni* may affect chronic herpesvirus control, as has been shown in an animal model [23]. Furthermore, we observed an increased risk of KSHV seropositivity with increasing IgE to *S. mansoni* adult worm antigen (SWA) and *S. mansoni* egg antigen (SEA) and IgG to SWA. We observed no association with *S. mansoni* IgG4 antibody levels with KSHV seropositivity. The (Th) 2 immune response to *S. mansoni,* characterised by IgE, IL4 and IL5 production has been linked to protection from *S mansoni* reinfection, while IgG4 production has been linked to susceptibility to reinfection [24]. This protective immunity tends to increase with age and requires repeated exposure to develop. The rationale is that the repeated exposure to antigens, for example from dead worms due to age or treatment, leads to immune boosting, leading to higher, and therefore protective,

levels of immunity [24]. Therefore, increased IgG and IgE antibody responses to *S. mansoni* could be used as a measure of repeated exposure to the parasite. The association of higher *S. mansoni* antibody concentration with KSHV seropositivity may be due to repeated exposure to the parasite, leading to interference with KSHV immunity and increasing KSHV antibody levels. Furthermore, the lack of association with *S. mansoni* IgG4 antibody concentrations may imply that the effect of *S. mansoni* on KSHV is through specific immune responses.

The disparity in KSHV prevalence between rural and urban areas also requires further investigation. A number of factors including environmental, behavioural and biological factors may explain this. For example, being chronically infected with infections such as malaria and helminths may affect KSHV transmission; these infections may be more common in rural areas than urbanized areas due to higher socioeconomic status, higher education levels and easier access to medical care in urban centres. Another example is local plant derivatives ("oncoweeds") that reactivate KSHV [25] that may be more common and frequently used in rural areas compared to urban areas. Behavioural factors affecting salivary exchange (for example children are more likely to share food on the same plate) may be more common in rural places compared to urbanized areas. Education levels are lower in Ugandan villages than in urban areas, and higher parental education has been associated with less risk of being KSHV seropositive in children [11, 12]. This association might be attributed to a number of factors including social behaviour, health care access, parasite infections among others.

Males tended to have higher KSHV prevalence and higher viral load in saliva, compared to females. This result has also been shown previously [26, 27]. Males are also at a higher risk of KS compared to females, especially among HIV negative persons [28, 29]. Others have also shown a higher KSHV load in blood in males compared to females [30]. Sex differences have been observed in other infections and disorders [31]. The general observation is that

there is a higher risk of infectious diseases (such as *Mycobacteria tuberculosis*, malaria and West Nile virus) in males compared to females, whereas there is a higher risk of autoimmune disease (such as Addison's disease and systemic lupus erythematosus) in females compared to males [31]. The risk of Burkitt's lymphoma is also higher in boys compared to girls [32-34]. These differences have been attributed to the higher responsiveness of immune cells in females compared to males, which protects females from infectious diseases, but also then predisposes females to autoimmune diseases. Some of the possible mechanisms responsible for sex differences in immunity include genetics, steroid hormones [31], and environmental exposures that are gender specific, although the precise mechanisms for sex-related immune differences are still to be elucidated. In relation to KSHV, the knockdown of androgen receptor expression in cultured human endothelial cells was associated with dysregulation of KSHV infection [35]. These findings require further investigations.

The effect of age of infection on disease outcomes may vary depending on the underlying mechanism. For example, in western countries, EBV infection in adulthood is associated with infectious mononucleosis [14], whereas early infection with EBV in Africa is associated with a higher viral load and risk for Burkitt's lymphoma [13]. Similarly, we observed that early infection with KSHV was associated with higher subsequent antibody levels, which were maintained for several years. Increased antibody titres to KSHV have been associated with the risk of KS [36]. In addition, the fact that children have more detectable KSHV DNA in both saliva and in blood compared to adults implies that children fail to control KSHV effectively. Uganda is a high disease burden setting. Multiple infections (and vaccines) in childhood may mean that "resources" for the immune system are prioritized to acute infections and vaccines, leading loss of control of chronic viruses such as KSHV. These two scenarios are not mutually exclusive, and may, in fact, have a cumulative effect. Further work on assessing immune exhaustion in children would be an obvious approach to take in

assessing the impact of multiple infections and vaccines, and susceptibility to KSHV infection.

Prevention of viral shedding in the saliva is paramount for blocking transmission, while deterrence of viral reactivation in the blood may prevent oncogenesis. We observed no correlation between levels of KSHV DNA in saliva and in blood. This is contrary to EBV, in which EBV DNA detection in blood has been correlated to that in saliva, with an increased risk of detecting EBV in blood if it was present in saliva [37]. Oral replication of γherpesviruses seems to be a paramount source of the virus [38]. The lack of correlation between oral and blood KSHV DNA levels is intriguing and requires further investigation. Perhaps, after primary infection, the virus spreads from the oral cavity to other parts of the body, establishing infections at different sites. Thereafter, the virus is controlled by different mechanisms at different sites – mucosal immunity in the oral cavity versus systemic immunity in other parts of the body. It is therefore important to investigate KSHV immunity in saliva to understand factors that affect viral shedding in saliva.

The lack of KSHV cellular immune dominance was reported previously [39, 40]. However, the limitations of previous studies are that they either used very few peptide pools or were not from KSHV endemic areas. Here we used peptide pools spanning the entire KSHV genome and observed a similar lack of immune dominance. Some immunodominance has been observed in other herpesviruses including EBV and HCMV [16, 41]. In KSHV, we have observed very low cell-mediated responses during early infection. The higher cellular immune responses in adults may result from recurrent antigen expression during reactivation. These responses may be HLA haplotype-specific as they tend to vary in different individuals. It would, therefore, be interesting to investigate HLA haplotypes in relation to KSHV epitopes. We also observed age group-specific (young adults) correlation of cellular-mediated immune responses with lack of detectable KSHV DNA in blood. This is suggestive that robust cellular immune responses are associated with reduced viral

reactivation. This result requires confirmation with an increased number of participants. Therefore, we have planned to test more samples for KSHV IFN-γ cellular responses. These responses seemed to be lost as people get older, probably due to a number of factors including immunosenescence and chronic inflammation seen in older people. It is not surprising given the wide range of complications which become apparent in older people [31]. This reduction in cellular responses as people get older may explain why KS and other KSHV malignancies are more common in older people.

In the current study, we have observed lower KSHV prevalence in those on HIV treatment and/or had CD4 counts above 500cells/μl, but this should be interpreted with caution. One might expect higher KSHV prevalence in HIV infected people due to their immunosuppressed state, although this was not what we observed. The immunosuppression could lead to the reactivation of KSHV in those already infected, leading to increased antibody levels, that could easily be detected by the serology assay. Similarly, the immunosuppression could render the few KSHV uninfected individuals susceptible to KSHV infection. Since most KSHV infections occur in childhood prior to horizontal HIV acquisition, reactivation is more likely to occur. One of the limitations of this finding was that we could not ascertain the exact treatment regimens or in some cases, whether the study participants were treated or not (we, therefore, used CD4 counts in those cases). Secondly, there was a significant number of individuals who were not tested for HIV. Therefore, although these data are intriguing, we cannot make definitive conclusions. Nevertheless, ART is well known for the rapid KS tumour resolution and prevention of oncogenesis when initiated. This is attained through recovery of immune function and suppression of both HIV viremia and KSHV blood viral load [42]. Furthermore, prevention of KSHV acquisition by ART in children has been observed [43], and the *in vitro* inhibitory effects of HIV protease inhibitors on KSHV replication have been documented [44]. These factors may suggest a possible effect of ART on KSHV, preventing acquisition (viral establishment) or reactivation of the virus.

Strengths and limitations

The particular strengths of this thesis were the large sample size, and different cohorts, allowing definitive epidemiological association between KSHV and various risk factors. In addition, we were able to assess KSHV prevalence and associated risk factors in population-based studies with a wide age range in various populations rather than relying on small high-risk populations of hospital patients.

Nevertheless, there were a few limitations in the current studies. The KSHV cellular immune response study (shown in chapter seven) used a small sample size, making definitive conclusions about the data difficult. However, the data are intriguing and testing more samples will confirm, or not, our initial observations. With the exceptions of the retrospective study, which determined the age of infection with KSHV, the rest of the studies were cross-sectional. This prevented confirmation of causation and makes cohort studies with appropriate follow up, necessary to confirm these findings. All the cross-sectional studies had minimal missing data, although the retrospective study (shown in chapter three), had some missing data. However, results from a sample of fewer participants with all consecutive data were analysed separately and the conclusions did not differ from the ones with missing data. Additionally, data were missing completely at random, suggesting there was at least no bias.

Recommendations and future proposed work

The incidence of KS is highest in resource-limited countries of Africa. Ultimately, with limited resources to manage cancer in Africa, prevention of KSHV transmission to uninfected individuals, and prevention of cancer development in already infected individuals would provide the the best strategies to improve public health. Prevention of transmission and disease progression can be achieved using two approaches. The first approach is to

understand factors affecting viral shedding in saliva because the reduction of viral shedding in saliva will prevent transmission to uninfected individuals. The second approach is to understand factors favouring viral control to prevent cancer development. To understand factors affecting viral shedding in saliva more, we plan to measure KSHV-specific IgA in oral fluids in those individuals from rural Uganda in whom we already have salivary viral load data, to ascertain the role of local immunity on viral control at mucosal sites. In order to understand factors that favour viral control in blood, we will test more samples for KSHV cellular responses to confirm (or not) the current ELISPOT findings. It is possible that if these immune responses could be provoked in high-risk KSHV-infected individuals (perhaps with a therapeutic vaccine), that this may impact on KSHV viral load and subsequent risk of KS.

In this current PhD research, we have observed a number of associations from cross-sectional studies which require confirmation of causation through cohort studies. Therefore, in further postdoctoral work, we plan to recruit 6-month-old children from the GPC and follow them for three years to look at the effect of malaria parasitaemia, as well as malaria treatment on KSHV acquisition and reactivation. We will also investigate immune mechanisms for any associations.

Other achievements

During this PhD, I contributed to work on other projects including studies on KSHV, Norovirus and Tuberculosis [45-48]. I also trained to perform KSHV specific assays which were developed at Viral Oncology Section at NIH and I was responsible for transfer and establishment of these assays at the MRC/UVRI and LSHTM Uganda Research Unit. Additionally, I facilitated the transfer of equipment required for the assays to the MRC Unit, as well as installation, validation and maintenance. I also took a leadership role in

establishing a field immunology laboratory in Kyamulibwa for sample processing in the field including PBMC isolation.

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Appendix 1: Ethical approval letters

UNHRO
Uganda National Health Research Organisation

Uganda Virus Research Institute

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REPUBLIC OF UGANDA

Our Ref: GC/127/16/09/566
Your Ref:

September 14, 2016

Ms. Angella Nalwoga,

RE: UVRI REC review of protocol titled **"Determinants and immune correlates of Kaposi's sarcoma associated herpesvirus (KSHV) suppression and transmission in Uganda."**

Thank you for submitting the response to the queries addressed to you by the UVRI REC.

This is to inform you that your response dated September 1, 2016 was reviewed and met the requirements of the UVRI REC.

UVRI REC annual approval has been given for you to conduct your research up to September 14, 2017. Annual progress report and request for extension should be submitted to UVRI REC prior to the expiry date, to allow timely review.

The reviewed and approved documents included;

1. UVRI REC Application form
2. Study Protocol.
3. Consent and assent forms
4. Information sheets
5. Questionnaires
6. Applicant's CVs

You can now continue with your study after registration with the Uganda National Council for Science and Technology (UNCST).

Note: UVRI REC requires you to submit a copy of the UNCST approval letter for the above study before commencement.

Yours sincerely,

[redacted]

Mr. Tom Lutalo
Chair, UVRI REC
C.C Secretary, UVRI REC

UGANDA VIRUS RESEARCH INSTITUTE ENTEBBE
RESEARCH ETHICS COMMITTEE
APPROVED
VALID UNTIL
14 SEP 2017
P. O. BOX 49, ENTEBBE
UGANDA

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: HS 2123 **25th October 2016**

Angela Nalwoga
Principal Investigator
MRC/UVRI Uganda
Research Unit on AIDS
Entebbe

Re: Research Approval: **Determinants and Immune Correlates of Kaposi Sarcoma Associated Herpesvirus Suppression and Transmission in Uganda**

I am pleased to inform you that on **11/10/2016**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period **11/10/2016** to **11/10/2019.**

Your research registration number with the UNCST is **HS 2123**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project.

As Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the research protocol or the consent form (where applicable) must be submitted to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local REC for review with copies to the National Drug Authority.
4. Unexpected events involving risks to research subjects/participants must be reported promptly to the UNCST. New information that becomes available which alters the risk/benefit ratio must be submitted promptly for UNCST review.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. A progress report must be submitted electronically to UNCST within four weeks after every 12 months. Failure to do so may result in termination of the research project.

LOCATION/CORRESPONDENCE

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COMMUNICATION

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WEBSITE: http://www.uncst.go.ug

Below is a list of documents approved with this application:

	Document Title	Language	Version	Version Date
1.	Research Protocol	English	1.0	8th July 2016
2.	Information Sheet Adult Consent	English and Luganda	1.1	10th October 2016
3.	Information Sheet Assent for Children 8 to 17 Years	English and Luganda	1.1	10th October 2016
4.	Information Sheet Mother's Consent	English and Luganda	1.1	10th October 2016
5.	Information Sheet Consent for Children 1-17 Years	English and Luganda	1.1	10th October 2016
6.	Information Sheet/Consent for Storage	English and Luganda	1.0	8th July 2016
7.	KSHV Study, 10-14 Years Olds and their Mothers	English and Luganda	N/A	N/A
8.	MRC/UVRI KSHV Study Questionnaire for GPC	English and Luganda	N/A	N/A

Yours sincerely,

[redacted]

Hellen N. Opolot
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Copied to: Chair, Uganda Virus Research Institute, Research Ethics Committee

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Observational / Interventions Research Ethics Committee

Ms Angela Nalwoga
LSHTM

30 November 2016

Dear Angela,

Study Title: Determinants and Immune correlates of KSHV suppression and transmission in Uganda

LSHTM Ethics Ref: 11881

Thank you for responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Protocol / Proposal	KSHV_study_protocol	08/07/2016	01
Protocol / Proposal	KSHV_Questionnaire_emabs	08/07/2016	01
Protocol / Proposal	KSHV_Questionnaire_GPC	08/07/2016	01
Information Sheet	EngV1_EMaBS_assent	08/08/2016	1
Information Sheet	EngV1_EMaBS_consent	08/08/2016	1
Information Sheet	EngV1_EMaBS_storage	08/08/2016	1
Information Sheet	EngV1_GPC_assent	08/08/2016	1
Information Sheet	EngV1_GPC_consent_adults	08/08/2016	1
Information Sheet	EngV1_GPC_consent_for_children	08/08/2016	1
Information Sheet	EngV1_GPC_storage	08/08/2016	1
Investigator CV	CV_for_Angela_Nalwoga_130616	05/09/2016	01
Investigator CV	CV for Robert Newton	05/09/2016	01
Investigator CV	Denise Whitby CV	05/09/2016	01
Investigator CV	Steve_cose_Academic CV	05/09/2016	01
Local Approval	UVRI_REC_approval	14/09/2016	01
Local Approval	KSHV AN Initial UNCST Approval	25/10/2016	1
Information Sheet	EngV1.2_EMaBS_assent	20/11/2016	1.2
Information Sheet	EngV1.2_EMaBS_consent	20/11/2016	1.2
Information Sheet	EngV1.2_EMaBS_consent_for_children	20/11/2016	1.2
Information Sheet	EngV1.2_EMaBS_storage_children_assent	20/11/2016	1.2

Information Sheet	EngV1.2_EMaBS_storage_consent_for_children	20/11/2016	1.2
Information Sheet	EngV1.2_EMaBS_storage_mother_consent	20/11/2016	1.2
Information Sheet	EngV1.2_GPC_assent	20/11/2016	1.2
Information Sheet	EngV1.2_GPC_consent_adults	20/11/2016	1.2
Information Sheet	EngV1.2_GPC_consent_for_children	20/11/2016	1.2
Information Sheet	EngV1.2_GPC_storage__consent_assent	20/11/2016	1.2
Information Sheet	EngV1.2_GPC_storage_consent_for_children	20/11/2016	1.2
Protocol / Proposal	KSHV_study_protocol_V1.2	20/11/2016	1.2
Covering Letter	response_to_LSHTM_ethics_21nov16	21/11/2016	1

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

Professor John DH Porter
Chair

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Improving health worldwide

UNHRO
Uganda National Health Research Organisation

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REPUBLIC OF UGANDA

Our Ref: GC/127/17/04/*317*
Your Ref:

April 4, 2017

Dear Prof. Alison Elliott

RE: UVRI REC review of protocol amendment for study titled **"Lake Victoria Island Intervention Study on Worms and Allergy-related diseases (LaVIISWA)" Amendment Appendix K.** A study to examine the prevalence of Kaposi's sarcoma associated herpesvirus (KSHV) and related viruses.

Thank you for submitting the above amendment dated March 15, 2017 to the UVRI Research Ethics Committee (REC).

This amendment was reviewed and met the requirements of the UVRI Research Ethics Committee. UVRI REC approval has been given for you to continue with the proposed amendment.

The reviewed and approved amendment is;

- Approval of investigation of Kaposi sarcoma associated herpesvirus and related viruses among 4210 individuals from LaVIISWA using stored samples.

You can continue with your study and remember to notify Uganda National Council for Science and Technology (UNCST).

Yours sincerely,

[redacted]

Mr. Tom Lutalo
Chair, UVRI REC
C.C Secretary, UVRI REC

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Observational / Interventions Research Ethics Committee

Prof Alison Elliott
Professor of Tropical Medicine
Department of Clinical Research (CRD)
LSHTM

28 April 2017

Dear Prof Alison

Study Title: Lake Victoria Island Intervention Study on Worms and Allergy-related diseases

LSHTM Ethics Ref: 9917 - 9

Thank you for your application for the above amendment to the existing ethically approved study and submitting revised documentation. The amendment application has been considered by the Interventions Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above amendment to research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval for the amendment having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Other	2. Protocol_LaVIISWA version 10, changes highlighted	12/03/2017	10
Other	3. LaVIISWA_Appendix K_protocol amendment Jan 2017_final	12/03/2017	2

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

[redacted]

Professor John DH Porter
Chair

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